The role of cancer related inflammation, Src Family Kinases and Matrix Metalloproteinase 9 in colorectal cancer

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Abstract

Colorectal cancer (CRC) is the third most common cancer in the UK with 41,000 new cases diagnosed in 2011. Despite undergoing potentially curative resection, a significant amount of patients develop recurrence. Biomarkers that aid prognostication or identify patients who are suitable for adjuvant treatments are needed. The TNM staging system does a reasonably good job at offering prognostic information to the treating clinician, but it could be better and identifying methods of improving its accuracy are needed.

Tumour progression is based on a complex relationship between tumour behaviour and the hosts' inflammatory responses. Sustained tumour cell proliferation, evading growth suppressors, resisting apoptosis, replicative immortality, sustained angiogenesis, invasion & metastasis, avoiding immune destruction, deregulated cellular energetics, tumour promoting inflammation and genomic instability & mutation have been identified as hallmarks. These hallmarks are malignant behaviors are what makes the cell cancerous and the more extreme the behaviour the more aggressive the cancer the more likely the risk of a poor outcome.

There are two primary genomic instability pathways: Microsatellite Instability (MSI) and Chromosomal Instability (CI) also referred to as Microsatellite Stability (MSS). Tumours arising by these pathways have a predilection for specific anatomical, histological and molecular biological features. It is possible that aberrant molecular expression of genes/proteins that promote malignant behaviors may also act as prognostic and predictive biomarkers, which may offer superior prognostic information to classical prognostic features.

Cancer related inflammation has been described as a 7^{th} hallmark of cancer. Despite the systemic inflammatory response (SIR) being associated with more aggressive malignant disease, infiltration by immune cells, particularly $CD8^{+}$ lymphocytes, at the advancing edge of the tumour have been associated with improved outcome and tumour MSI. It remains unknown if the SIR is associated with tumour MSI and this requires further study.

The mechanisms by which colorectal cancer cells locally invade through the bowel remain uncertain, but connective tissue degradation by matrix metalloproteinases (MMPs) such as MMP-9 have been implicated. MMP-9 has been found in the cancer cells, stromal cells and patient circulation. Although tumoural MMP-9 has been associated with poor survival, reports are conflicting and contain relatively small sample sizes. Furthermore, the influence of high serum MMP-9 on survival remains unknown.

Src family kinases (SFKs) have been implicated in many adverse cancer cell behaviors. SFKs comprise 9 family members BLK, C-SRC, FGR, FYN, HCK, LCK, LYN, YES, YRK. C-SRC has been the most investigated of all SFKs, but the role of other SFKs in cellular behaviors and their prognostic value remains largely unknown. The development of Src inhibitors, such as Dasatinib, has identified SFKs as a potential therapeutic target for patients at higher risk of poor survival. Unfortunately, clinical trials so far have not been promising but this may reflect inadequate patient selection and SFKs may act as useful prognostic and predictive biomarkers.

In chapter 3, the association between cancer related inflammation, tumour MSI, clinicopathological factors and survival was tested in two independent cohorts. A training cohort consisting of n=182 patients and a validation cohort of n=677 patients. MSI tumours were associated with a raised CRP (p=0.003). Hypoalbuminaemia was independently associated with poor overall survival in TNM stage II cancer (HR 3.04 (95% CI 1.44 – 6.43);p=0.004), poor recurrence free survival in TNM stage III cancer (HR 1.86 (95% 1.03 – 3.36);p=0.040) and poor overall survival in CI colorectal cancer (HR 1.49 (95% CI 1.06 – 2.10);p=0.022). Interestingly, MSI tumours were associated with poor overall survival in TNM stage III cancer (HR 2.20 (95% CI 1.10 – 4.37);p=0.025).

In chapter 4, the role of MMP-9 in colorectal cancer progression and survival was examined. MMP-9 in the tissue was assessed using IHC and serum expression quantified using ELISA. Serum MMP-9 was associated with cancer cell expression (Spearman's Correlation Coefficient (SCC) 0.393, p<0.001)) and stromal expression (SCC 0.319, p=0.002). Serum MMP-9 was associated with poor recurrence-free (HR 3.37 (95% CI 1.20 – 9.48);p=0.021) and overall survival (HR 3.16 (95% CI 1.22 – 8.15);p=0.018), but tumour MMP-9 was not survival or MSI status.

In chapter 5, the role of SFK expression and activation in colorectal cancer progression and survival was studied. On PCR analysis, although LYN, C-SRC and YES were the most highly expressed, FGR and HCK had higher expression profiles as tumours progressed. Using IHC, raised cytoplasmic FAK (tyr 861) was independently associated with poor recurrence free survival in all cancers (HR 1.48 (95% CI 1.02 – 2.16);p=0.040) and CI cancers (HR 1.50 (95% CI 1.02 – 2.21);p=0.040). However, raised cytoplasmic HCK (HR 2.04 (95% CI 1.11 – 3.76);p=0.022) was independently associated with poor recurrence-free survival in TNM stage II cancers. T84 and HT29 cell lines were used to examine the cellular effects of Dasatinib. Cell viability was assessed using WST-1 assay and apoptosis assessed using an ELISA cell death detection assay. Dasatinib increased T84 tumour cell apoptosis in a dose dependent manner and resulted in reduced expression of

nuclear (p=0.008) and cytoplasmic (p=0.016) FAK (tyr 861) expression and increased nuclear FGR expression (p=0.004).

The results of this thesis confirm that colorectal cancer is a complex disease that represents several subtypes of cancer based on molecular biological behaviors. This thesis concentrated on features of the disease related to inflammation in terms of genetic and molecular characterisation. MSI cancers are closely associated with systemic inflammation but despite this observation, they retain their relatively improved survival. MMP-9 is a feature of tissue remodeling during inflammation and is also associated with degradation of connective tissue, advanced T-stage and poor outcome when measured in the serum. The lack of stromal quantification due to TMA use rather than full sections makes the value of tumoural MMP-9 immunoreactivity in the prognostication and its association with MSI unknown and requires further study. Finally, SFK activation was also associated with SIR, however, only cytoplasmic HCK was independently associated with poor survival in patients with TNM stage II disease, the group of patients where identifying a novel biomarker is most needed. There is still some way to go before these biomarkers are translated into clinical practice and future work needs to focus on obtaining a reliable and robust scientific technique with validation in an adequately powered independent cohort.

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Declaration

The work presented in this thesis was performed entirely by the author except as acknowledged. This thesis has not been previously submitted for a degree or diploma at this or any other institution.

Arfon G M T Powell

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Publications

Published Papers

1: Mansouri D, **Powell AG**, Park JH, McMillan DC, Horgan PG. Long-Term Follow-Up of Patients Undergoing Resection of TNM Stage I Colorectal Cancer: An Analysis of Tumour and Host Determinants of Outcome. World J Surg. 2016; 40(6):1485-91. PubMed PMID: 26920405.

2: Park JH, **Powell AG**, Roxburgh CS, Horgan PG, McMillan DC, Edwards J. Mismatch repair status in patients with primary operable colorectal cancer: associations with the local and systemic tumour environment. Br J Cancer. 2016; 114(5):562-70. PubMed PMID: 26859693

3: Park JH, McMillan DC, **Powell AG**, Richards CH, Horgan PG, Edwards J, Roxburgh CS. Evaluation of a tumor microenvironment-based prognostic score in primary operable colorectal cancer. Clin Cancer Res. 2015; 21(4):882-8. PubMed PMID: 25473000.

List of Abbreviations

5-FU	5-Flurouracil
AI	Apoptosis index
APC	Adenomatous Polyposis coli
CIMP	CpG Island Methylator Phenotype
CI or CIN	Chromosomal Instability
CT	Computed Tomography
ELISA	Enzyme Linked Immune-Substrate Assay
FAK	Focal Adhesion Kinase
FAP	Familial Adenomatous Polyposis
FFPE	Formalin Fixed Paraffin Embedded
Hb	Haemoglobin
H&E	Haematoxylin and Eosin
HLA	Human Leukocyte Antigen
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
HS	Horse Serum
IGF-1	Insulin like Growth Factor-1
IHC	Immunohistochemistry
LNR	Lymph Node Ratio
mGPS	Modified Glasgow Prognostic Score
MSI	Microsatellite Instability
MSS	Microsatellite Stability
MMP-9	Matrix metalloproteinase 9
MRI	Magnetic Resonance Imaging
ON	Overnight
PCR	Polymerase Chain Reaction
PI	Proliferation Index
PtI	Petersen Index
RT-PCR	Real-time PCR
SA	Serrated Adenocarcinoma
SFK	Src Family Kinases
TMA	Tissue Microarray
TME	Total Mesorectal Excision

TUNEL	Terminal deoxynucleotidyl transferase dUTP Nick End Labelling
WST-1	Water Soluble Tetrazolium Salts-1

1. Introduction

1.1 Biomarkers and cancer – the clinical challenge

To treat any illness appropriately requires some idea of its severity, which guides management. Between 1942 and 1956 Professor Pierre Denoix devised the Tumour Nodes Metastasis (TNM) staging system for solid tumours, which stratifies patients into discrete staging groups (Brierley, 2006). The anatomically based TNM staging system offers a useful ordinal parameter of outcome, however its predictive value while valuable needs to be improved. The TNM staging system only offers an anatomical snapshot of the disease and does not sufficiently take into account an individual's cancer behaviour. Therefore, there is a continuing need to develop new tests that offer better prognostic and predictive value in order to help guide overall management.

Molecular biomarkers in the context of cancer research are molecules occurring in body fluids or tissues in association with cancer. Biomarkers can be objectively measured and evaluated as an indicator of normal biological processes, pathological processes, or pharmacological responses to a therapeutic intervention (Ludwig, 2005). A perfect cancer biomarker would be reproducible, cost-effective, sensitive and specific for diagnosis, correlate with disease severity and predict response to treatment. Unfortunately, developing good biomarkers is difficult and no marker so far identified covers all these areas. Even the more limited challenge to develop and validate good biomarkers that help identify higher-risk patients requiring adjuvant therapies, and predict responsiveness to such therapies, is formidable.

1.2 The hallmarks of cancer

Cancer is a disease of uncontrolled growth and proliferation of cells. As abnormal cells multiply they may spread to distant organs and form secondary tumours. Cancer cells invade and break down adjacent tissue, damaging normal function and may ultimately lead to death.

Malignant cells have specific behavioral traits that allow them to be distinguished from the normal cell. In summarizing many years of work, Hanahan and Weinberg identified sustained proliferation, evading growth suppressors, resisting apoptosis, replicative immortality, sustained angiogenesis, invasion & metastasis, avoiding immune

destruction, deregulated cellular energetics, tumour promoting inflammation and genomic instability & mutation as hallmarks of cancer (Hanahan, 2011).

These cancer hallmarks are caused by dysregulation of normal molecular processes. Tumour aggressiveness varies widely not only between different entities but between the same tumour type in different patients, which results in different outcomes: some patients have long disease free survival but in others recurrence, local or distant, are ultimately fatal. Identifying patients likely to have a poor outcome, who will benefit most from assertive adjuvant treatment and surveillance is a clinical priority. Identifying and accurately quantifying aberrant expression of molecules involved in regulating the cancer 'hallmark' behaviours might offer superior prognostic information to classical prognostic features such as morphology (independently or together).

1.3 Classes of Cancer Biomarkers

1.3.1 Diagnostic markers

Diagnostic biomarkers help a clinician to determine if a disease is present in a symptomatic patient. In addition, they may also be useful in screening asymptomatic healthy people. An ideal diagnostic biomarker should have high sensitivity and specificity (>99.9%) for the disease under question. (Sensitivity is the proportion of people who have the disease and who have a positive test, whereas specificity is the proportion of people without the disease who have a negative test). For a biomarker to be incorporated into routine clinical practice it must also be cost-effective, reproducible and relatively non-invasive.

In colorectal cancer, the gold standard diagnostic process remains a multimodal assessment of radiology, endoscopy and histological assessment of biopsy samples with immunohistochemistry if needed. The serum concentration of carcinoembryonic antigen is sometimes determined, but may be raised in adenocarcinomas arising in any site, may not be raised in early colorectal cancer, and may show variation with carcinoma differentiation. Its sensitivity and specificity are poor (Duffy, 2001).

1.3.2 Prognostic markers

A prognostic biomarker provides information about the malignant potential of a tumour including who may develop recurrence after surgery or help identify patients who will have a short survival time. Prognostic markers in current clinical use include the hormone

receptors oestrogen receptor (ER) and progesterone receptor (PR) in breast cancer, the cell proliferation marker Ki67 in neuroendocrine tumours and the human epidermal growth factor receptor 2 (Her2) in breast cancer (Slamon, 2001) and gastric cancer (Bang, 2010).

Although there is a vast literature relating to prognostic biomarkers, much of it is of poor quality, with technical limitations including poor reproducibility, and many underpowered studies. Very few prognostic biomarkers have been established in routine clinical practice. Even now, the best available prognostic biomarker in colorectal cancer remains TNM stage. Future prognostic markers will need to be equally good or better on their own merits, or offer enhanced prognostication within specific TNM stage subgroups.

1.3.3 Predictive markers

Following identification of genetic aberrations and upregulation of signaling pathways as prognostic biomarkers, new treatments aimed to antagonise these biologically active processes have been developed. Examples include ER in breast cancer (Harvey, 1999) and HER2 in both breast (Slamon, 2001) and gastric cancer (Bang, 2010). However, oestrogen deprivation for breast cancer was established long before the identification of ER.

In colorectal cancer, the only predictive biomarker currently used is wild-type K-ras, which identifies patients likely to respond to Cetuximab, a monocloncal antibody directed against epidermal growth factor receptor, in patients with metastatic cancer who have not responded to chemotherapy (Karapetis, 2008). Currently, there are no predictive biomarkers available for patients who have undergone potentially curative resection but are considered to require adjuvant therapy, which represents an unmet clinical need.

1.3.4 Identification and validation of diagnostic, prognostic and predictive biomarkers

Many publications examine associations between expression of various proteins and survival in nearly every human cancer. Unfortunately, even controlling for any specific cancer type and individual protein studied, different studies have such varied methods for quantification and reporting outcomes that establishing a robust evidence base for translation into clinical practice is difficult. In an attempt to standardise development, measurement and validation of biomarkers, The Biomarker Definitions Working Group (2001) gave a formal definition of a biomarker, in the hope that this would result in

internationally accepted markers, and facilitate their incorporation into routine clinical practice.

The pathways proposed for identifying and validating these different types of biomarker follow five conceptual phases described by Pepe *et al*.

1.3.4.1 Identification and validation of screening biomarkers

The process of identification and validation has five conceptual phases (Pepe, 2001):

Phase 1: Preclinical exploratory

Two processes underpin discovery of novel biomarkers; hypothetico-deductive reasoning and molecular profiling of normal and cancer tissues. Molecular profiling identifies candidate markers by their differential expressions between normal and tumour. The hypothetico-deductive (theoretical) approach identifies potential biomarkers via a hypothesis based on current knowledge of cancer pathophysiology. High throughput genomic or proteomic studies examine differential expression of multiple genes. As a candidate biomarker progresses through these developmental phases, the number of patients in each phase increases. Although this initial stage utilises relatively few samples, significant resource are needed to optimise marker quantifcation and establish robust and reproducible methodologies.

Phase 2: Clinical assay development and validation

Phase 1 only proposes a preferred method for molecular quantification, which requires independent validation. Phase 2 aims to confirm the optimum methodology for molecular quantification and reporting. A good technique should be relatively simple to perform and reproducible. Using a range of statistical tests, various analytical and reporting methods are associated with the presence of disease in appropriately characterised patient cohorts.

Phase 3: Retrospective longitudinal study

Archival material (e.g. stored blood) from cancer patients, prior to their diagnosis of cancer, may be examined for biomarker expression and compared with control patients, to evaluate a biomarker's ability to diagnose preclinical disease or increased risk. This phase is of particular interest if interval screening is thought to be potentially useful. In some instances, it is not possible or appropriate to perform this stage, and validation studies may enter at or jump to a different phase.

Phase 4: Prospective screening studies

The sensitivity and specificity of the biomarker is determined in a relevant population. The frequency of disease stages detected during the screening test period also offers valuable information on the potential impact of cancer associated mortality. However, direct associations with survival are not studied at this point. To achieve adequate detection of cancers, this phase will require a large number of participants, which also acts as a general feasibility study in preparation for phase 5.

Phase 5: Cancer control studies

Phase five assess whether there is an associated reduction of cancer burden on the population. This relates to cancer mortality and costs of screening and treatment per life saved. An ideal screening biomarker will detect disease at an early stage, reduce mortality and be cost effective. Despite appropriately powered prospective studies it can be difficult to determine how much 'if any' benefit the population derives.

1.3.4.2 Identification and validation of prognostic biomarkers

Initial phase: Preclinical, exploratory

Similar to the Pepe phase 1, biomarker identification is either based on hypothetico-deductive reasoning (i.e. theoretical considerations) or high throughput molecular profiling. Molecular quantification is validated across different scientific techniques to ensure consistent expression. For example, biomarkers based on mRNA copy numbers should undergo immunohistochemistry to confirm proportional aberrant protein expression.

Intermediate phase: Clinical assay development and validation

Further molecular quantification is conducted assessing the technical methodology against a series of endpoints and tumour characteristics. The biomarker assay is finalised with clearly specified reagents and reducible methodology. Using a training cohort, usually retrospective, different molecular quantification thresholds are associated with survival data and tumour characteristics such as stage or grade.

Final phase: Independent validation

Validation of the biomarker assay is performed in an independent cohort with appropriate statistical power. To minimise bias, the best study conditions for biomarker validation is a prospective randomised control trial. If statistical power is sufficient, this will offer prognostic stratification in the whole cohort, in different tumour stages and in patients receiving different treatments. Such an independent validation study usually requires a large number of patients, probably recruited in multiple centers.

1.3.4.3 Identification and validation of predictive biomarkers

Identification and validation of biomarkers predictive of therapy response depends on the type of treatment. Treatments can be broadly grouped into cytotoxic therapies and biological therapies. Cytotoxic therapies are cytotoxic (more or less) to all living cells whereas biological therapies are directed against specific physiological processes and may have a more favourable therapeutic profile.

Preclinical exploratory phase

Predictive biomarker development is synergistic with drug discovery. Once the contribution or relevance of a biomarker to the malignant phenotype is confirmed, there is a prospect of targeted therapies to counteract the malignant process. Initial laboratory work may confirm reduced expression across different quantification techniques including polymerase chain reaction (PCR), western blotting or immunohistochemistry, or reduction of downstream target activity. Once inhibition of the biomarker is confirmed, mechanistic studies aim to confirm biomarker-mediated reversal or amelioration of the malignant phenotype. An appropriate biomarker assay is developed which includes clearly specified reagents and a reproducible technical methodology. The targeted therapy progresses through drug discovery and validation.

Clinical trial stage

Once a targeted treatment regimen and linked biomarker assay ‘companion diagnostics’ have been formalised, an adequately powered prospective randomised control trial is required to examine the efficacy of the drug in improving response over current standard-of-care chemotherapy with stratification of outcomes by biomarker expression. This stage usually requires many patients treated in multiple centers. Initial patient selection often involves people who have metastatic disease who have not responded well to standard oncological therapy.

1.4 Colorectal cancer

1.4.1 Anatomy and physiology of the normal colorectum

The colorectum (colon and rectum) is the mucosa-lined muscular tube forming the most distal part of the digestive tract, distal to the ileocaecal valve and proximal to the anal canal. Its chief function is to absorb water as digested food passes through, and store faeces until elimination is appropriate. It is ~1.5 meters long and derives from the embryological mid- and hindgut. Its embryological origin determines its blood supply and lymphatic drainage. Nine anatomical areas are conventionally recognised: caecum, ascending colon, hepatic flexure, transverse colon, splenic flexure, descending colon, sigmoid colon, rectosigmoid and rectum. The proximal colon (caecum to splenic flexure) is of midgut origin and receives blood from the superior mesenteric artery. The distal colon, of hindgut origin (splenic flexure to anus) receives blood from the inferior mesenteric artery (figure 1.1). The extent of surgical resections for colorectal cancer are determined by the blood supply of the area of the colorectum where the tumour is located. For simplicity the colorectum can also be broken down into only three segments: (Figure 1.1)

- Right colon – Caecum, ascending colon, hepatic flexure, transverse colon and splenic flexure
- Left colon – Descending and sigmoid colon
- Rectum – Rectosigmoid junction and rectum

Colorectal cancer can occur anywhere in the colorectum, but most (69%) arise within the descending colon, sigmoid colon, rectosigmoid junction and rectum (Figure 1.1, Phillips, 2014). Two thirds of the remaining cancers occur in the caecum and ascending colon.

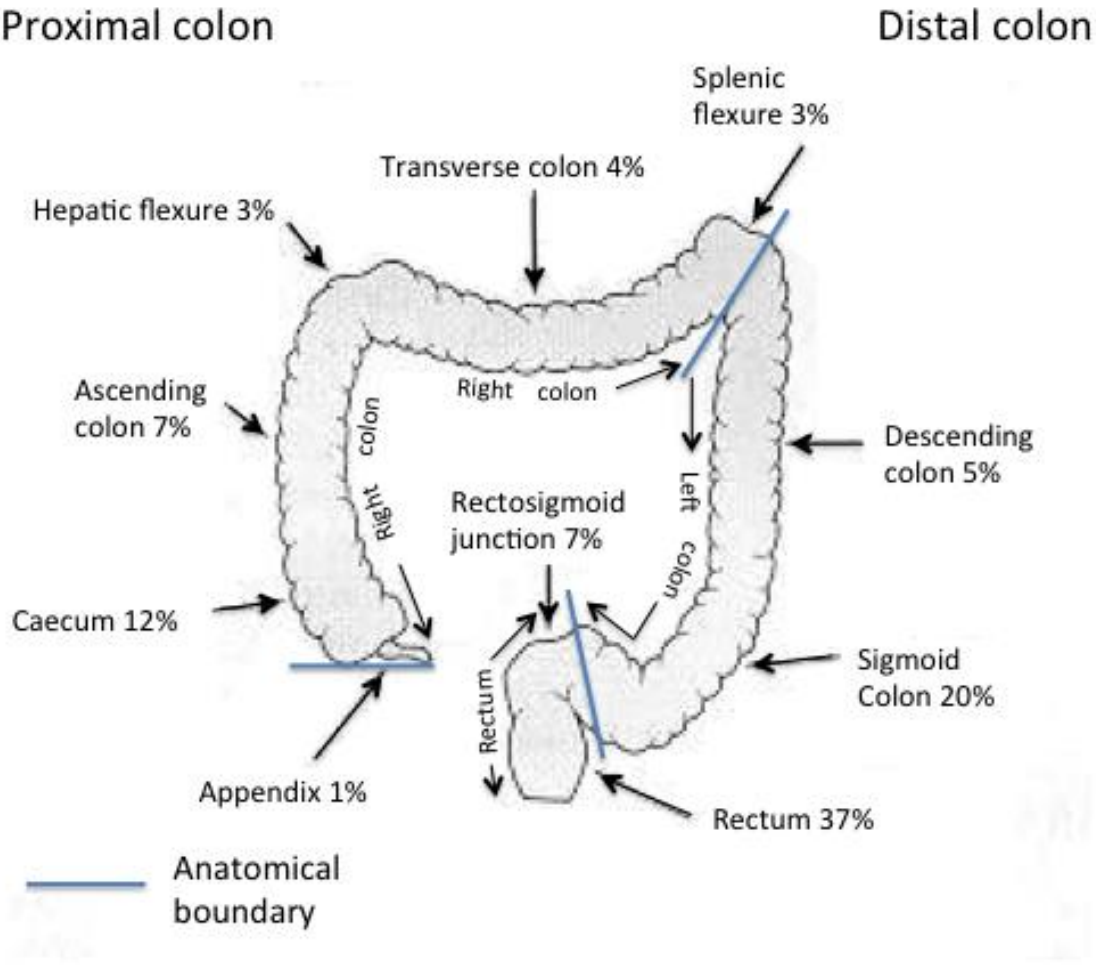


Figure 1.1: Distribution of colorectal tumours throughout the colorectum *(Adapted from (Austoker J. BMJ 1994 309:382)*

1.4.2 Normal histology of the colorectum

Histologically the colorectum is continuous, with no obvious boundary or structural differences between colon and rectum. It is made up of layers comprising from the lumen outwards; mucosa, lamina propria, muscularis mucosa, submucosa, muscularis propria, subserosa and serosa. These structures are shown in figure 1.2.

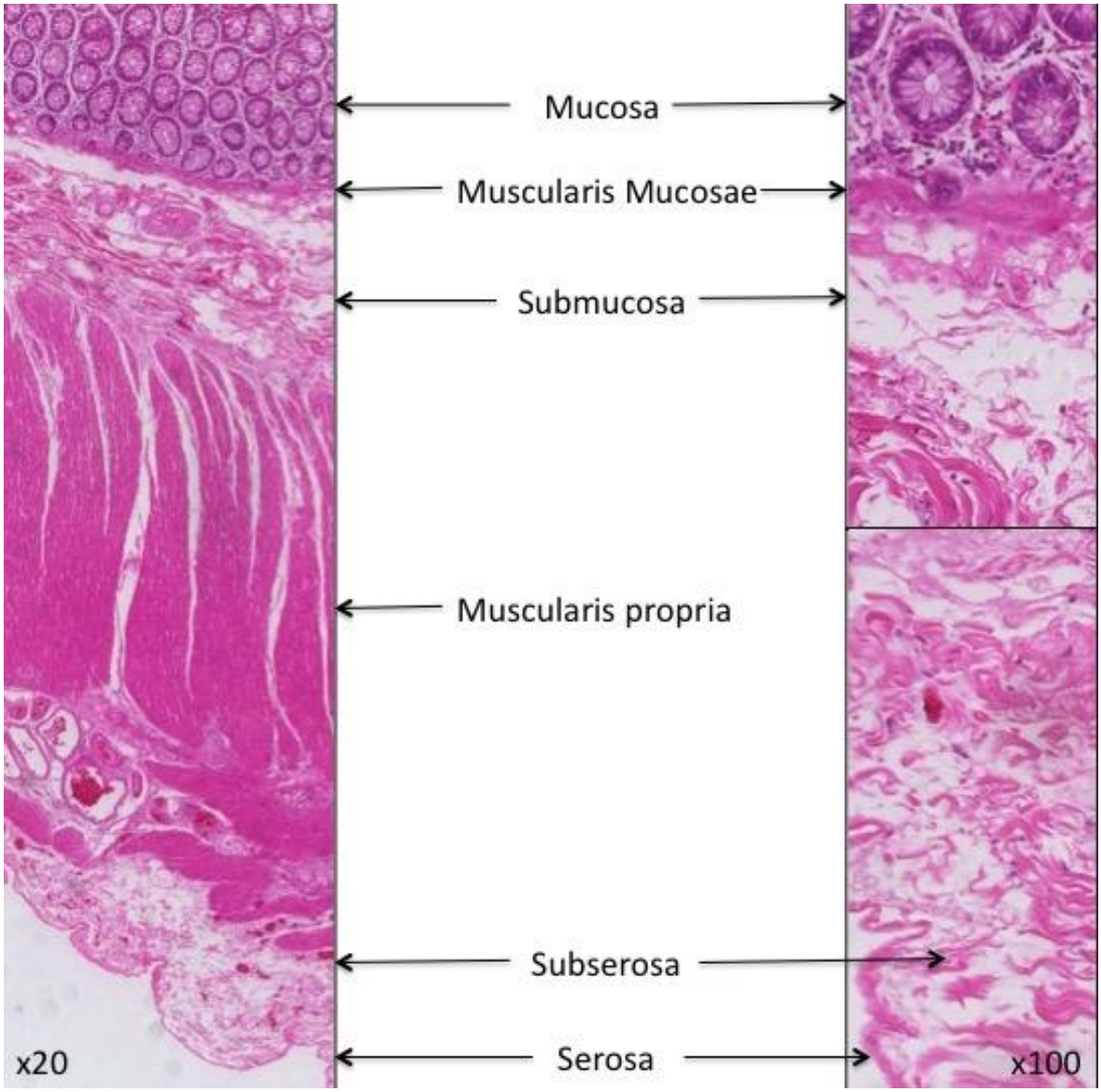


Figure 1.2: Normal colorectum demonstrating the histological layers

On the left a x20 magnification demonstrates an overview of the layers. Top right, at x100 magnification, demonstrates the relationship between mucosa, muscularis mucosae and sub serosa. Note the circular crypt profiles containing epithelial cells within the mucosa. Bottom right demonstrates, at x100 magnification, the subserosa and serosa.

The mucosa is made up of crypts comprising columnar epithelium (enterocytes), goblet cells and supportive connective tissue. At the bottom of these crypts are crypt stem cells that generate new epithelial cells. Cells migrate up the crypt towards the luminal surface where they slough off or undergo apoptosis. A typical crypt is demonstrated in figure 1.2 above.

1.4.3 Epidemiology of colorectal cancer

1.4.3.1 Incidence and survival

Colorectal cancer is the third most common cancer in the UK, with 41,000 new cases diagnosed in 2011, accounting for 12.5% of all new cases of cancer in general. Incidence rates are approximately 75 / 100,000 per annum in men and 59 / 100,000 in women. Incidence rates have remained on the whole static over the last twenty years. Although survival rates have doubled (Cancer Research UK, 2014), overall five-year survival is still relatively poor at only 50% in men and women (Cancer Research UK, 2014). In 2006, the NHS Bowel Cancer Screening Programme was introduced in England. This has resulted in earlier cancer detection and a 16% reduction in deaths related to colon cancer has followed (Cancer Research UK, 2014). Despite screening and better colorectal cancer treatment, many patients still die of their cancer.

1.4.3.2 Aetiology of colorectal cancer

Most colorectal cancers are sporadic. Only about 5-10% occur in the setting of a defined hereditary cancer syndrome, but about 20% of all colorectal cancers are thought to arise in patients with some component of family risk (Lynch, 2003). Unlike some other cancers, no specific aetiological factors have been identified, but lifestyle factors do appear to play an important role (Parkin, 2011; WCRF/AICR, 2010). The risk of developing colorectal cancer is increased in people who are inactive, have an increased body mass index, smoke tobacco and drink larger volumes of alcohol. Diets rich in red meat and low in fibre have also been associated with higher risk of colorectal cancer development (WCRF/AICR, 2010).

1.4.3.3 Hereditary colorectal cancer

The two major forms of hereditary colorectal cancer are familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC) or Lynch syndrome. A diagnosis of hereditary colorectal cancer is based on family history, multiple primary cancers, age of onset of the cancer and specific phenotypic features (Lynch, 2003). FAP usually presents with many colonic polyps and this widespread dysplastic transformation is a result of a germ-line mutation in the adenomatous-polyposis-coli (APC) gene on chromosome 5. HNPCC, which is the most common cause of hereditary colorectal cancer, does not present as a polyposis. It is caused by a germ-line mutation in one or other of the

DNA mismatch repair (MMR) genes (hMLH1, hMSH2, hMSH6, PMS1 and PMS2) and is also associated with extra-colonic tumours notably of the endometrium, ovary, stomach, small bowel, pancreas, hepatobiliary, brain and the upper uroepithelial tract (Vasen, 1999). The microsatellite instability pattern of genetic injury associated with HNPCC is also seen in some sporadic cancers, which may be either microsatellite unstable (MMR deficient) or microsatellite stable (associated with APC mutation). The molecular genetic basis of sporadic colorectal cancer will be discussed later.

1.4.4 Colorectal cancer pathology

1.4.4.1 Histological subtypes of colorectal cancer

Colorectal cancers are named according to their presumed cell of origin and pattern of differentiation. Adenocarcinomas, presumed to arise from glandular tissue, are much the most common, accounting for about 95% of all cancers. Other malignant tumours (ie cancers) include carcinoids (now called neuroendocrine tumours), sarcomas and lymphomas.

It is now widely accepted most (if not all) colorectal adenocarcinoma result from the neoplastic progression of pre-malignant polyps or adenomas. This sequence of events has been named the adenoma-carcinoma sequence (Muto, 1975; Day, 1978). Below is a figure demonstrating a carcinoma with nearby adenoma (figure 1.3), from which it is presumed to have arisen.

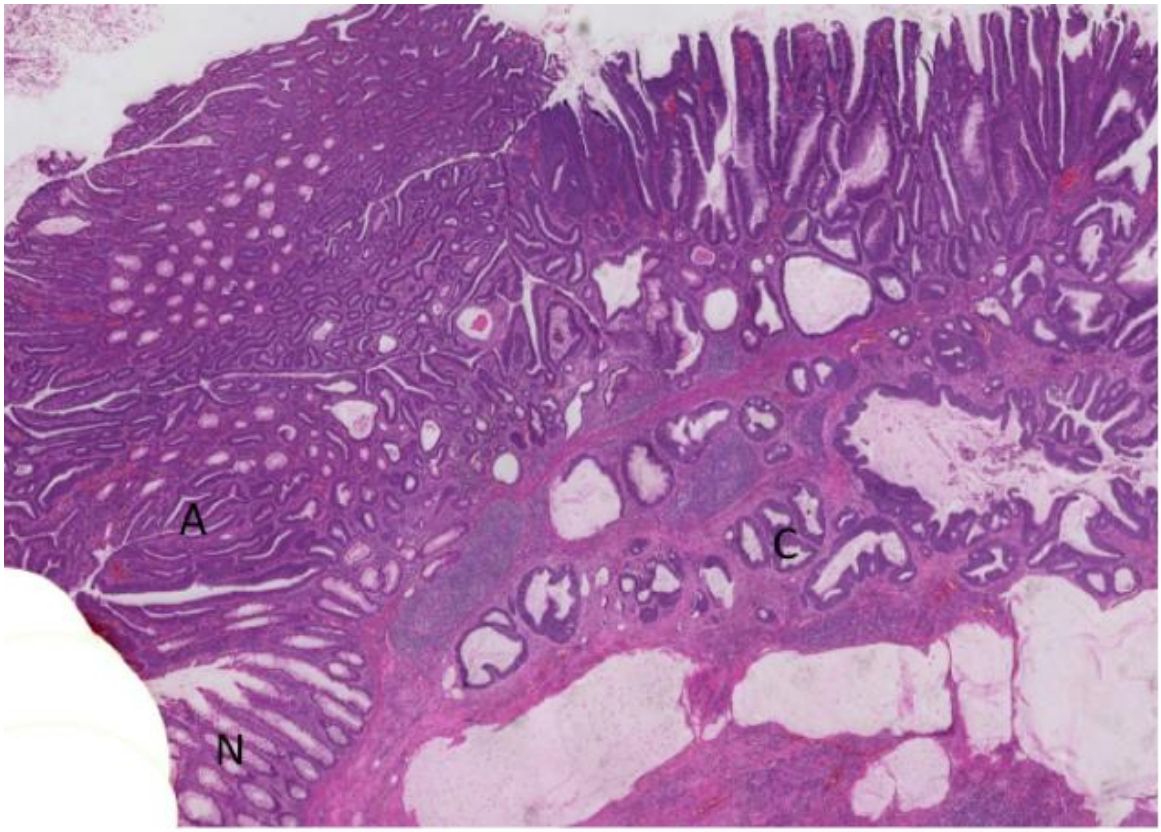


Figure 1.3: H+E demonstrating normal colonic mucosa, adenoma and adenocarcarcinoma

H+E section showing normal (N), adenoma (A) and invasive cancer (C)

Apart from confirmation of the diagnosis of adenocarcinoma, additional histopathological features reported during assessment of a colorectal cancer excision specimen include differentiation, mucin secretion, necrosis, lymphatic and venous invasion in addition to T stage and nodal involvement, all of which may offer prognostic information.

1.4.4.2 Differentiation

Differentiation describes how closely the malignant glands resemble normal colorectal glandular tissue. Given the association with loss of cellular adhesion, poorly differentiated adenocarcinomas have been associated with more advanced tumour stage and poorer outcome (Halvorsen, 1988). While various grading systems have been suggested, the Royal College of Pathologists favour a simple dichotomy into well differentiated versus moderate/poor differentiation. Poorly differentiated tumours are defined by 'irregularly folded, distorted and often small tubules or the absence of any tubular formation' (RCP datasets). Figure 1.4 demonstrates carcinomas with moderate and poor differentiation.

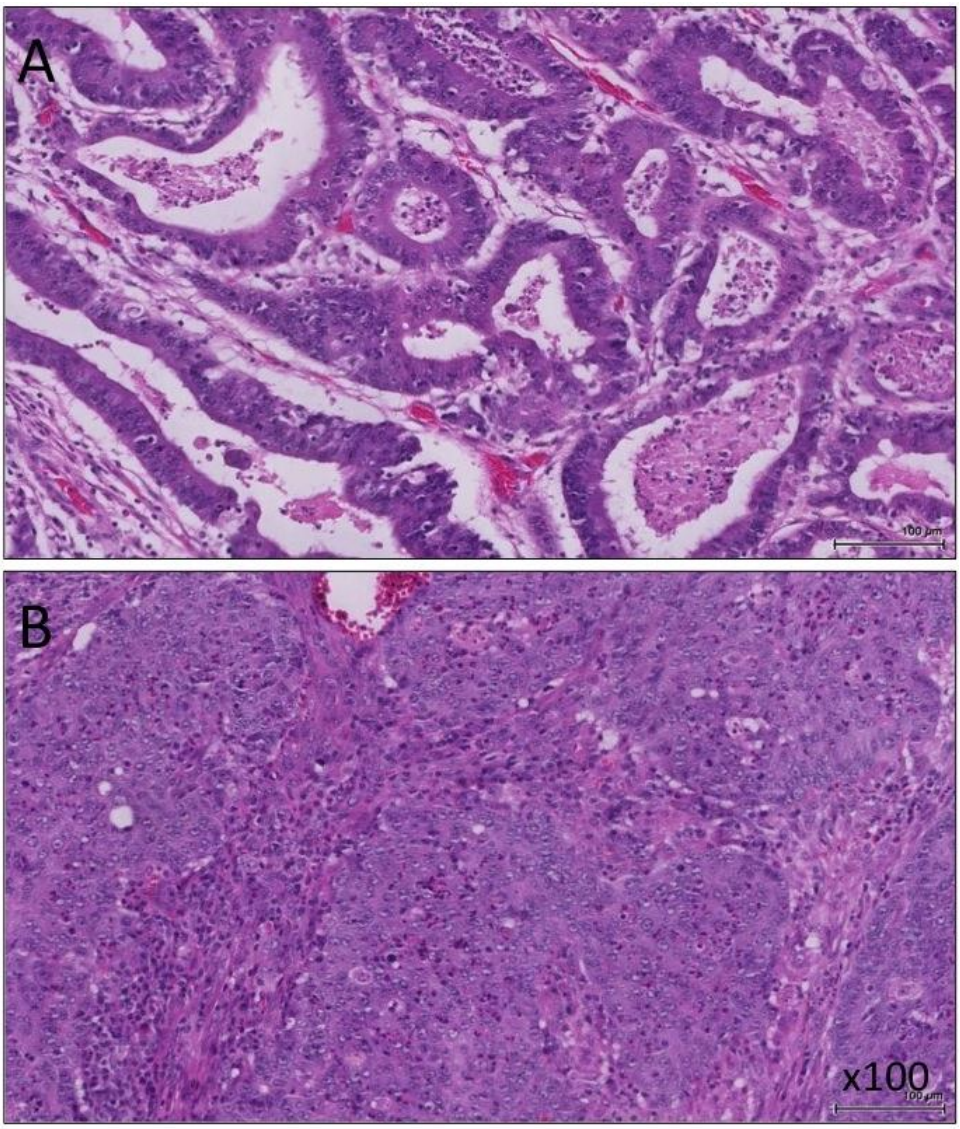


Figure 1.4: H+E demonstration of moderate and poor differentiation

A.) H+E slide shown at x100 magnification demonstrating moderate differentiation. Definite glandular acini are seen.
B.) H+E shown at x100 magnification demonstrating poorer differentiation. There are no acini.

Categorising differentiation into three categories (well, moderate, poor) aims to achieve an ordinal categorical variable describing tumour aggressiveness. While the figure above may seem to demonstrate a clear difference in differentiation, in clinical practice such distinctions are difficult. Interobserver agreement is generally accepted as poor (Chandler, 2008) and tumour histology is often heterogenous within the same specimen and thus correlation with clinical endpoints may not be robust due to vagaries of specimen sampling. These limitations mean that tumour differentiation is not a core dataset item for planning adjuvant treatment during MDT discussions.

1.4.4.3 Mucin production

Extracellular mucin accumulates in about 18% of all colorectal cancers (Hogan, 2014). The exact mechanism underlying this phenomenon remains unclear. The World Health Organisation (WHO) define mucinous carcinoma as one with >50% of its mass composed of extracellular mucin pools (Hamilton, 2000). Figure 1.5 shows a focally mucinous adenocarcinoma.

Historically, mucinous adenocarcinoma has been associated with poorer prognosis and advanced stage (Purdie, 2000; Verhulst, 2012). Despite consensus reporting guidelines, research studies continue to show significant methodological and reporting variability. Along with interobserver variability in pathological reporting and potential misrepresentation in pathological sections, imprecisely defined association studies makes it difficult to translate this pathological variable into a routinely used parameter for treatment planning and it does not form a core data item for MDT discussion.

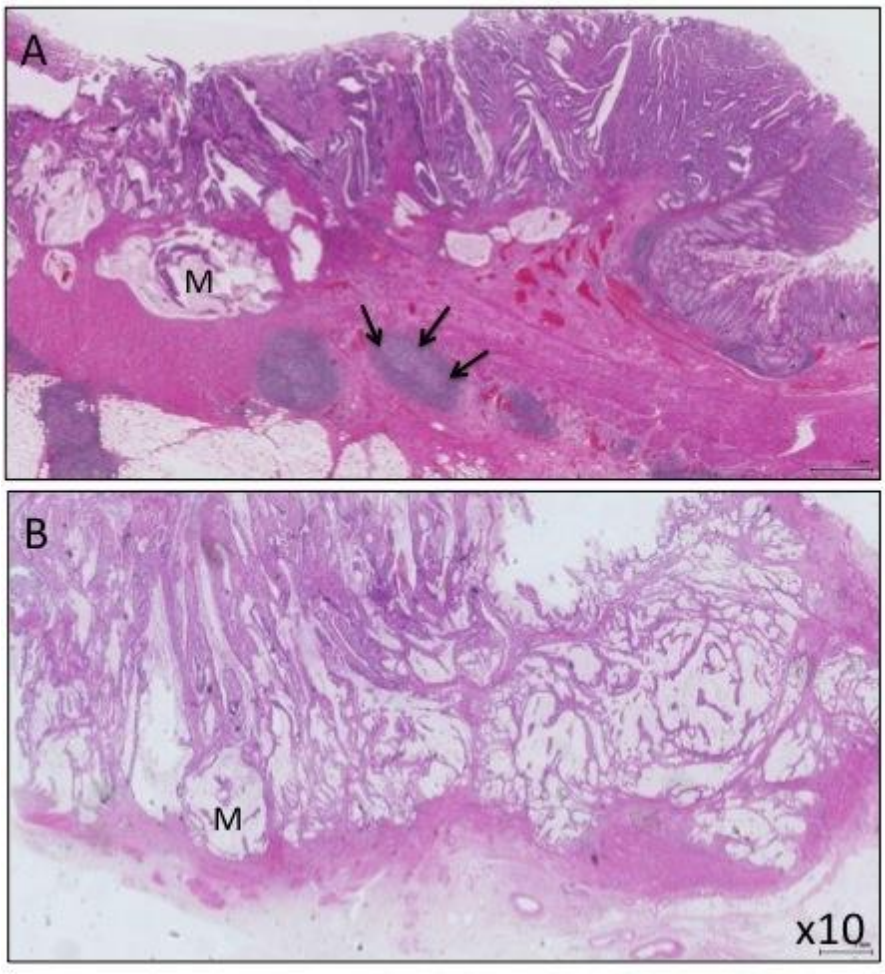


Figure 1.5: Demonstration of different degrees of tumour mucin

H+E sections shown at x10 magnification demonstrating A.) Focal mucin production in a MSI tumour; A 'Crohn's disease like' inflammatory reaction (arrowed) is present. B.) Extensive mucin production

1.4.4.4 Tumour necrosis

Tumour necrosis common in solid tumours and classically attributed to ischaemic injury due to highly proliferative tumours outstripping their blood supply (Pollheimer, 2010). Extensive tumour necrosis has also been associated with advanced tumour stage and poorer survival (Gao, 2005; Pollheimer, 2010; Richards, 2012). Figure 1.6 below demonstrates different tumours with variable degrees of necrosis. Like some other pathological variables, poor interobserver reproducibility and ill-defined thresholds mean it has not been established as a core pathological data item for adjuvant treatment planning in colorectal cancer.

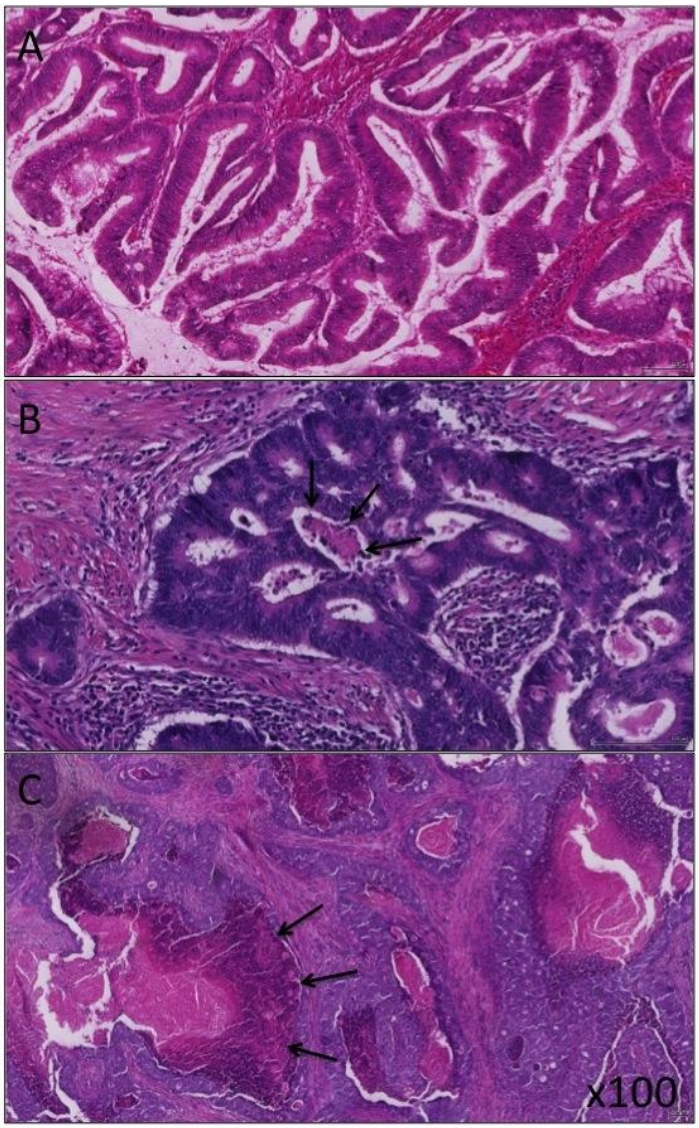


Figure 1.6: Demonstration of different degrees of tumour necrosis

H+E sections shown at x100 magnification demonstrating A.) No necrosis, B.) Focal necrosis and C.) Extensive necrosis. Necrotic areas are arrowed.

1.4.4.5 Extramural venous invasion

Elastin staining for the assessment of vascular invasion increases detection rates from about 1 in 5 to 3 in 5 (18% to 58%);(Roxburgh, 2010a). RCPath guidelines describe venous invasion as 'tumour present within an extramural endothelium-lined space that is either surrounded by a rim of muscle or contains red blood cells'. Extramural vascular invasion, since the introduction of elastin staining, has been shown to be a stage-independent predictor of poor survival (Roxburgh, 2010). Figure 1.7 demonstrates venous invasion in a normal H+E and an elastic H+E staining.

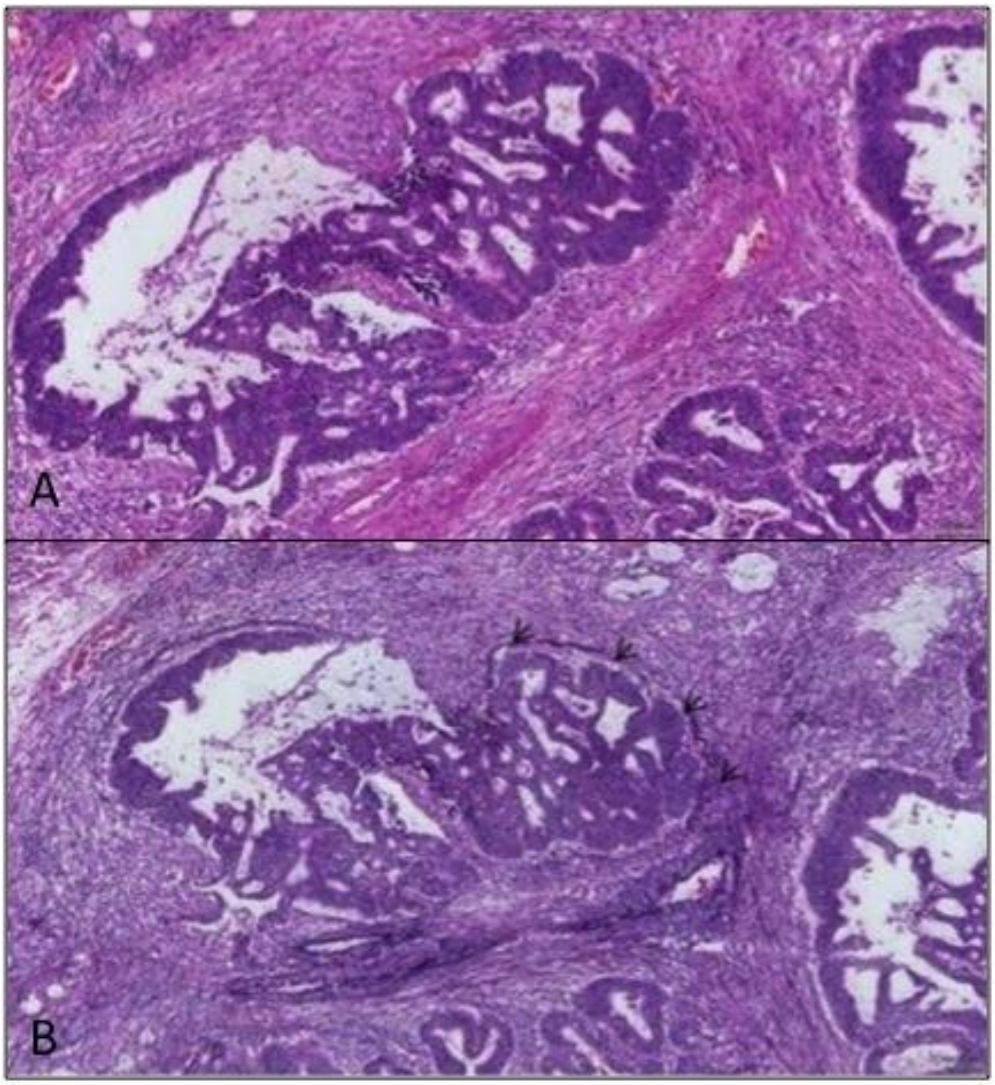


Figure 1.7: H+E demonstration of extramural vascular invasion

A.) H+E section shown at x100 magnification with a cancer gland next to an artery. B.) Elastic H+E shown at x100 magnification of the same tumour area as shown in A. Note the vascular elastic lamina surrounding the cancer gland (arrowed).

1.4.4.6 Histological grading systems

Optimal cancer treatment maximises survival and minimises morbidity. This is a difficult challenge as different patients require different treatments but criteria for making treatment choices are imperfect. Unlike breast and prostate cancer, which have well specified grading systems, a reliable grading system in colorectal cancer has proven difficult. TNM staging and venous invasion remain the best available prognostic biomarkers. Histological reporting bias remains a problem but alternatives including automated reporting of immunohistochemistry, ELISA and PCR while in theory they might be expected to offer greater reliability and objectivity present many challenges of their own and have yet to supersede histological data, which turns out to be surprisingly robust.

1.4.5 Molecular pathology of colorectal cancer

1.4.5.1 Genomic instability and colorectal cancer

The adenoma-carcinoma hypothesis of colorectal cancer

This term refers to the pathway from normal epithelium, via adenoma formation and eventual malignant transformation to an established cancer. It is supposed that these transformations are caused by sequential accumulation of genetic (and epigenetic) injuries. This hypothesis is supported by the observation that many patients with colorectal cancer also have adenomas present in the resected specimen (either separate, or closely associated with the carcinoma and from which the carcinoma may have arisen). Patients with carcinoma are on average 5 years older than patients with adenomas, in keeping with a model of progression from adenoma to carcinoma (Muto, 1975). In 1990, Fearon and Vogelstein proposed their famous genetic model to account for this progression (Fearon, 1990). Figure 1.8 illustrates progression from normal epithelium to invasive adenocarcinoma and with some of the commonly associated genetic changes, including activation of oncogenes and inactivation of tumour suppressor genes.

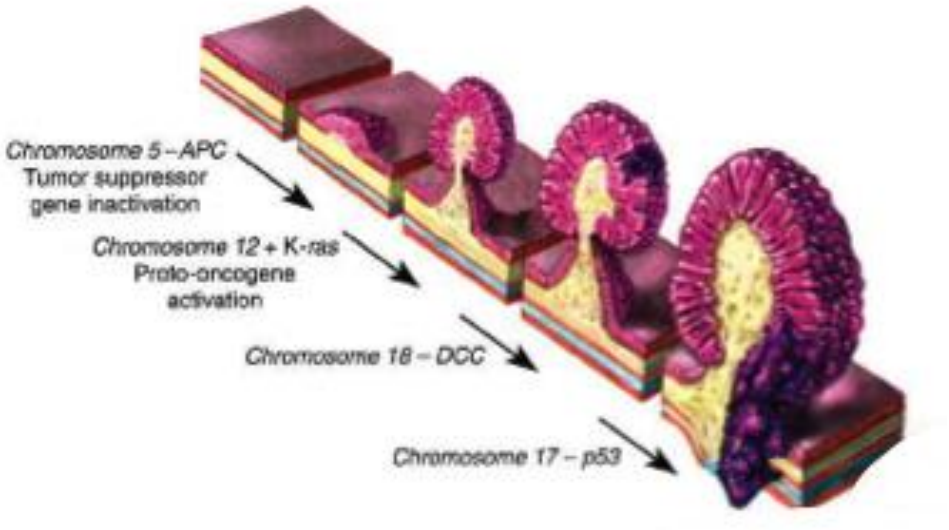


Figure 1.8: The adenoma carcinoma sequence and associated genetic events in tumours with chromosomal instability *(Adapted from Gordon 2007).*

It is now widely accepted that distinct molecular pathways including the chromosomal instability (CI) pathway (in which microsatellite tandem repeat sequences are usually unchanged) and the mutually exclusive microsatellite instability (MSI) pathway are associated with different patterns of mutation, pathological features and even survival in colorectal cancer.

1.4.5.2 Microsatellite stability and colorectal cancer

Molecular pathology of chromosomal instability

Overall the chromosomal instability pathway predominates, being present in about 85% of all colorectal cancers. It is characterised by allelic losses, chromosomal translocations and gene amplifications. While the exact mechanism that triggers the CI pathway remains unclear, certain oncogenes and tumour suppressor genes are commonly mutated in colorectal cancers.

The Adenomatous Polyposis Coli (APC) gene is commonly mutated in invasive cancers but mutations also characteristically occur prior to malignant tranformation. Powell (1992) reported a frequency of APC mutation nearly equal, at ~ 60%, in both adenomas and invasive cancers.

Activating K-*ras* mutations occurs in ~ 40% of adenomas and adenocarcinomas (Vogelstein, 1988). K-*ras* activating mutations are more commonly seen in larger adenomas when associated with concomitant APC mutations (Jen, 1994; Rashid, 1999). Since K-*ras* mutation occuring in both adenomatous and hyperplastic polyps (Kim, 2011),

it has been suggested that K-*ras* supports tumour growth while not playing a role in tumour initiation; but this is context dependent.

TP53 is a tumour suppressor gene located on the short arm of chromosome 17. It is commonly mutated in human cancers. It normally prevents cell proliferation in the presence of DNA damage, promotes DNA repair and apoptosis (Leslie, 2002). Altered p53 has been reported in a quarter of adenomas, half of polyp cancers and nearly three quarters of more advanced adenocarcinomas (Leslie, 2002) and a role for TP53 in the transition from pre-malignant polyps to cancer seems likely.

1.4.5.3 Microsatellite instability in colorectal cancer

Molecular pathology of microsatellite instability

Although very strongly associated with cancers in people with HNPCC, MSI also occurs in a non-trivial subset of sporadic colorectal cancers. MSI is present in 15% of all cancers and is characterised by frameshift mutations and base-pair substitutions. These substitutions are commonly found in the short tandem repeat DNA segments called microsatellites. Unlike the CI pathway, this destabilisation pathway is caused by mutations (or epigenetic silencing) or one or another of the DNA mismatch repair proteins mainly (MLH1, MSH2, MSH6 and PMS2). Instability of these 'microsatellite' short tandem repeats refers to the tendency of the number of tandem repeats to be altered as a consequence of 'slippage' of DNA polymerase and lends itself to detection by PCR techniques (Figure 1.9).

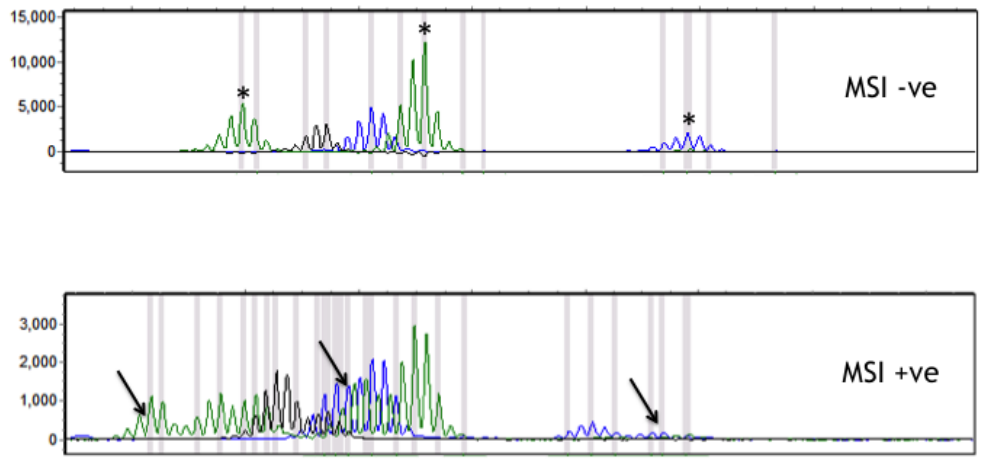


Figure 1.9: PCR demonstration of MSI compared with CIN from two different colorectal cancers

The MSI tumour peaks have a longer tail (arrows) compared with the MSS peaks (stars). This longer tail represents the accumulation of microsatellite repeats at these specific loci.

In contrast to HNPCC, in which loss of MMR function is due to mutation, sporadic MSI tumours are usually caused by hypermethylation of cytosine residues of the cytosine and guanine rich promoter sequences of MLH1 (Kane, 1997). This epigentic silencing leads to reduction or total loss of gene transcription. Cancers with high levels of CpG island methylation are said to have the 'CpG island methylator phenotype (CIMP; Toyota, 1999) and represent a clinically and aetiologically distinct group with 'epigenetic instability'. Loss of MMR protein expression results in alteration of microsatellites throughout the genome; this serves as a marker of ineffective mismatch repair and implies increased mutation of important tumour suppressor genes and oncogenes (including some which contain exonic microsatellites, like the TGF beta type II receptor, mentioned below).

Although it is generally accepted that CI colorectal cancer usually follows an adenoma-carcinoma sequence, the pathway to MSI colorectal cancer is less clear cut. Transforming growth factor-B (TGF-B) type II receptor is mutated in 90% of MSI colorectal cancers (Parsons, 1995). TGF-B RII is a tumour suppressor gene that regulates transcription of genes relating to cellular proliferation (Markowitz, 1995). Bcl-2-associated X protein (BAX) is inactivated in approximately 50% of all MSI tumours due to frameshift mutations and confers a cellular survival benefit through disruption of apoptosis mediated by Bcl-2 (Miquel, 2005; Trojan, 2004).

BRAF is a component of the RAS-RAF-MAPK signalling pathway, which mediates cellular responses to growth signals. BRAF mutation is strongly associated with MMR deficient CRC and is mutually exclusive with mutation of the K-*ras* oncogene (Rajagopalan, 2002; Yuen, 2002). Kambara *et al* (2004) observed that in sporadic MSI colorectal cancer, BRAF mutation was associated with the CpG island methylation phenotype. The association between BRAF mutation and sporadic MSI tumours was further strengthened by the observation that none of the 18 HNPCC tumours exhibited BRAF mutations (Kambra, 2004).

Pathological characteristics associated with microsatellite instability in colorectal cancer

MSI tumours occur more frequently in the proximal colon; in female patients; and are more likely to be larger, stage T3, but node negative (Soreide, 2006). Pathologically these tumours exhibit poorer differentiation, copious extracellular mucin production and lack the so-called 'dirty necrosis' characteristic of many colorectal cancers (Greenson, 2003). There is evidence that MSI tumours have a higher apoptosis to proliferation ratio, which may compensate for their increased cell proliferation activity (Michael-Robinson, 2001). There is also a pronounced peritumoral inflammatory infiltrate, which appears as a 'Crohn’s

disease like' inflammatory reaction (figure 1.5, page 50), as well as an increased density of $CD8^+$ lymphocytes within cancer cell nests (Dolcetti, 1999).

Prognostic value of microsatellite instability in colorectal cancer

Despite associations between MSI cancer and usually adverse prognostic factors such as more advanced T stage and a mucinous phenotype, these cancers have been associated with a better outcome. A systematic review and meta-analysis by Popat et al showed that in 32 studies of 7,642 patients, MSI tumours had a significantly better prognosis compared with their CI counterparts, with a hazard ratio for overall survival of 0.65 (95% CI, 0.59 to 0.71 (Popat, 2005). Despite this association with improved survival, MSI tumours do not appear to respond well to adjuvant 5-fluorouracil chemotherapy. A large study by Hong et al and meta-analysis by Guastadisegni et al (31 studies; 12,782 patients) concluded that 5-FU chemotherapy improved survival in CI colorectal cancer patients but not MSI patients (Guastadisegni, 2010; Hong, 2012). Little is known about clinicopathological factors predicting survival in patients with MSI tumours. Identifying such factors and pathophysiological processes underpinning their behaviour could suggest novel therapeutic targets for adjuvant biological therapies in patients with high risk MSI cancers.

1.5 Diagnosis and staging of colorectal cancer

1.5.1 Diagnosis

In the UK and elsewhere, patients with colorectal cancer are diagnosed via three main pathways. Unfortunately, almost one-third of patients present as an emergency with symptoms relating to advanced disease including obstruction, perforation or significant bleeding (Bass, 2009). For many of these patients, curative surgery is not an option and they undergo palliative treatment only. Patients with significant but less severe symptoms such as altered bowel habit, less significant bleeding and weight loss will be referred by a primary care physician as an urgent suspected cancer (USC). About half of these patients still have locally advanced disease or lymph node metastasis at presentation, with a higher risk of subsequent recurrence. To combat this issue, by 2010 the National Bowel Cancer Screening Programme had been established. Based on the faecal occult blood (FOB) test, patients in the age range of 50-74 are asked to submit a stool sample every 2 years. Patients with a positive FOB test are invited to undergo a screening colonoscopy by a certified screening endoscopist.

1.5.2 Staging and prognosis

1.5.2.1 Radiological staging

Staging allows rational treatment planning adapted to the likely prognosis of an individual patient. Unfortunately, because for most patients with metastatic carcinoma, surgery can only offer local symptomatic control (although this is very important for quality of life), pre-operative radiological staging is fundamental to identifying patients in whom there is a chance surgery may be curative. To find distant metastases, if present, patients with colon cancer undergo computed X ray tomography (CT) of chest, abdomen and pelvis. In rectal cancer, surgical resection presents greater technical challenges and so regional tumour staging is augmented by magnetic resonance (MRI) imaging of the pelvis.

1.5.2.2 Pathological staging

Colorectal cancer spread occurs locally by direct invasion through the bowel wall or systemically via lymphatic or venous invasion. Staging by pathological assessment of these anatomical / morphological parameters after surgical resection remains essential. There are 3 main pathological staging systems in clinical use; Dukes' stage, the modified Astler-Coller classification and the AJCC/UICC TNM (Tumour, Node, Metastasis) staging system. Each of these staging systems are largely based on a pathological assessment of tumour depth of invasion (T), the presence of lymph node metastasis (N) and the presence of distant metastases (M) (table 1.1 and figure 1.10).

Although Dukes' stage was for a long time the pathological staging system of choice, it was poor at risk stratifying patients within the local (Dukes' B) and lymph node metastasis group (Dukes' C). The Astler-Coller classification, a modification of Dukes' staging system, attempted to address these limitations by subcategorizing these groups into B1-3 and C1-3 by local tumour spread. A weakness of this approach is that T staging stratifies patients at risk of poor *local* control, whereas N stage stratifies patients at risk of poor *systemic* control, and the Astler-Coller classification inadequately stratifies patients with lymph node metastasis, as the subgrouping is based on degree of tumour penetration through bowel wall. TNM staging system aims to control for this by assessing the degree of local *and* lymph node spread' and offers the best form of pathological prognostication in routine clinical use. It is has undergone review many times over recent years and is

currently on its 7th edition with modifications based on emerging scientific evidence. In the case of colorectal cancer, TNM7 is not much different from TNM 5 and 6.

Table 1.1: The pathological components of the TNM staging system (7th edition)

TNM Component	**Pathological description**
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria
T1	Tumour invades submucosa
T2	Tumour invades muscularis propria
T3	Tumour invades through the muscularis propria and into the subserosa or non-peritonealised perirectal/pericolonic tissues
T4a	Tumour penetrates to the surface of the visceral peritoneum
T4b	Tumour directly invades or is adherent to other organs or structures
Nx	Lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis to 1-3 regional lymph nodes
N1a	Metastasis in 1 regional node
N1b	Metastasis in 2-3 regional nodes
N1c	Tumour deposits in the subserosa, mesentery, or nonperitonealized pericolic or perirectal tissues without regional nodal metastasis
N2	Metastasis to 4 or more regional lymph nodes
N2a	Metastasis in 4-6 regional lymph nodes
N2b	Metastasis in 7 or more regional lymph nodes
Mx	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis identified
M1a	Metastasis confined to one organ or site
M1b	Metastasis in more than one organ/site or the peritoneum

ANATOMIC STAGE/PROGNOSTIC GROUPS					
Stage	T	N	M	Dukes	MAC
0	Tis	N0	M0	–	–
I	T1	N0	M0	A	A
	T2	N0	M0	A	B1
IIA	T3	N0	M0	B	B2
IIB	T4a	N0	M0	B	B2
IIC	T4b	N0	M0	B	B3
IIIA	T1–T2	N1/N1c	M0	C	C1
	T1	N2a	M0	C	C1
IIIB	T3–T4a	N1/N1c	M0	C	C2
	T2–T3	N2a	M0	C	C1/C2
	T1–T2	N2b	M0	C	C1
IIIC	T4a	N2a	M0	C	C2
	T3–T4a	N2b	M0	C	C2
	T4b	N1–N2	M0	C	C3
IVA	Any T	Any N	M1a	–	–
IVB	Any T	Any N	M1b	–	–

Figure 1.10: Components of the different pathological staging systems (adapted from AJCC TNM 7th edition)

Dukes' – Dukes' staging system
MAC – modified Astler-Coller classification

As shown above, the pathological parameters are then combined to form the TNM stage. There are 4 main stages (I-IV) with I representing local disease, II locally advanced, III lymphatic spread to regional lymph nodes and IV the presence of distant metastasis. In addition there is an enhanced staging system represented by an alphabetical suffix A, B or C. In routine clinical practice and for the purposes of prognostication only the main groups are considered. Prognosis varies according to stage with 93% of patients with stage I

colorectal cancer living for 5 years compared to 6% of patients with stage IV disease (Cancer Research UK, 2014), (Table 1.2).

Table 1.2: Table summarising components of the Dukes' and TNM staging systems for colorectal cancer and 5-year survival (Cancer research UK)

TNM Classification	**T**	**N**	**M**	**5-year survival**	
Stage I	T1	N0	M0	93%	Confined to bowel wall
	T2	N0	M0		
Stage II	T3	N0	M0	77%	Locally advanced
	T4	N0	M0		
Stage III	T1, T2	N1-N2	M0	48%	Node positive disease
	T3, T4	N1-N2	M0		
Stage IV	Any T	Any N	M1	6%	Metastatic Disease

1.5.2.3 Limitations of the AJCC/UICC TNM staging system

TNM staging offers an anatomical snapshot of a dynamic and biologically active disease process, which integrates time and tumour biology. Although it offers an ordinal prognostic marker of survival, its predictive value in terms of identifying patients for adjuvant treatment or assessing if patients will respond to treatment is poor. A definite gap is that unlike breast and prostate cancer in which cancer grading makes a valuable contribution to prognostication, this is not the case in the current colorectal cancer TNM staging. Other limiting factors are inherent to the pathological processing of the tumour. Firstly, sections are simply representative and may under-stage the degree of penetration of the tumour through the bowel wall. Secondly, identifying all lymph nodes in a resected specimen can be challenging. The number of lymph nodes recovered varies widely between specimens. Some of this variation is probably real but pathologist diligence, neo-adjuvant treatment, scrupulousness in avoiding double counting, use of special fixatives (e.g.Carnoy's), and just how many lymphocytes are considered to constitute a lymph node all have an influence. Since lymph node staging is dependent on the number of lymph node containing carcinoma, non-identification of even one positive node may result in a different N staging. The introduction of a positive to total volume lymph node ratio tried to address this, but with conflicting results lymph node ratio has not become established in routine clinical practice. Despite these limitations highlighting the possibility of stage

migration or under-staging, TNM staging system is a useful ordinal predictor of outcome. Supplementing TNM stage with effective biomarkers seems to offer the best prospect of improved prognostication and for predicting response to adjuvant therapies.

1.6 Treatment options for colorectal cancer

1.6.1 Surgery

Surgery remains the primary modality for cure in colorectal cancer, but also has an important palliative role. The type of surgery performed depends on cancer location, pathological characteristics of the tumour and patient factors.

1.6.1.1 Elective surgery

Historically, surgical resection of colorectral cancer was performed via a large laparotomy incision; it is now more common to use laparoscopic techniques. The abdomen is imaged to confirm resectibility and to identify occult metastatic spread. Patients undergoing potentially curative resection will have an en bloc oncological resection with the intention of removing the tumour with adequate margins and also draining lymph nodes. Adequate lymphadenectomy requires an anatomical resection based on the arterial supply to the region of the colorectum containing the tumour.

Tumours of the right colon (caecum, ascending and transverse colon) are removed by right hemicolectomy, with ligation of ileocolic, right and middle colic arteries depending on tumour site. Tumours of the left colon (descending colon) are removed by left hemicolectomy with ligation of the left colic artery. Depending on precise site, tumours of the sigmoid colon are removed by sigmoid colectomy or anterior resection with ligation of the left colic artery and superior rectal artery. Depending on the level of the tumour and its proximity to the anus, rectal tumours are removed by anterior resection or abdomino-perineal resection (APR).

Over the past 25 years, oncological outcomes following rectal cancer surgery have improved with widespread adoption of total mesorectal excision (TME). T3 rectal tumours invade the mesorectum; TME requires anatomical dissection in the mesorectal plane. This improves the rate of clear margins with a reduction in the risk of local recurrence (Heald, 1986). The local recurrence rate following TME is reported as 4-5%, a reduction from 25% using previously accepted techniques (MacFarlane, 1993).

Some colorectal cancers can be excised locally at colonoscopy or transanally. Indications for potentially curative local excision include:

- Mobile tumours
- T1 tumours (assessed by ultrasonography)
- Well or moderately differentiated histology (determined by biopsy)
- Tumour size less than three centimetres.

1.6.1.2 Emergency Surgery

Despite better patient awareness and referral pathways, unfortunately 30% of patients still present as an emergency with obstruction, bleeding or perforation. Emergency presentation is a stage-independent adverse prognostic factor for short and long term oncological survival. Challenges remain, but improvements in operative technique and peri-operative care mean that most can be treated by a two stage (Hartmann's) procedure or resection and primary anastomosis. Following the SCOTIA trial, segmental resection is preferred to subtotal colectomy in obstructed distal colon cancers (SCOTIA, 1995). Segmental resection was associated with better long term GI function, but in the case of caecal perforation or synchronous tumours, subtotal colectomy remains the best option (SCOTIA, 1995).

1.6.2 Neoadjuvant therapy

TME is now standard of care in surgery for rectal cancer. Unfortunately, some patients still have margin-threatening disease and remain at higher risk of local recurrence. Therefore, patients with rectal cancer undergo pelvic MRI and, depending on the findings, may be offered short or long course radiotherapy or chemoradiotherapy.

1.6.2.1 Short course radiotherapy

Short course radiotherapy reduces local recurrence rates in patients undergoing TME (Folkesson, 2005; Kapiteijn, 2001). A dose of 25 Gy is given in 5 daily fractions over 1 week prior to definitive surgery. NICE guidelines suggest that patients with moderate risk tumours should receive short course radiotherapy. Moderate risk is defined as:

- T3 disease which is not margin threatening or
- Non-margin threatening lymph nodes
- Radiological evidence of extramural vascular invasion

1.6.2.2 Long course preoperative chemoradiotherapy

Patients receive 45 Gy of radiotherapy in 25 fractions over 5 weeks followed by an interval of 6-10 weeks to allow a response. Chemotherapy regimens are at the discretion of the oncologist. Historically these have largely been 5-FU based with good results (Sauer, 2004). However, newer regimens with Capecitabine are being studied (Gérard, 2010). NICE guidance suggests that patients with moderate or high risk tumours should receive long course chemoradiotherapy. High risk is defined as:

- A threatened (<1mm) or breached resection margin or
- Low tumours encroaching on the inter-sphincteric plane or with levator muscle involvement.

1.6.3 Adjuvant therapy

Following potentially curative surgery, adjuvant therapy aims to eradicate micrometastases thus increasing the rates of cure. Although the use of adjuvant chemotherapy in patients with stage II carcinomas is controversial (Benson, 2004), it is now widely accepted that stage III patients should be offered adjuvant treatment (Andre, 2009; NICE, 2011). Chemotherapeutic agents available for colorectal cancer patients include 5-Flurouracil (5-FU), Leucovorin (folinic acid), Oxaliplatin, Capecitabine and Irinotecan. These are often given as combinations including:

- FOLFOX - 5-FU, Leocovorin and Oxaliplatin
- FOLFIRI - 5-FU, Leucovorin and Irinotecan
- XELOX - Oxaliplatin and Capecitabine

Although historically patients with stage III disease were usually offered 5-FU, recent evidence suggests a further reduction in recurrence rates is possible with the addition of Oxaliplatin (FOLFOX) (Andre, 2009). Guidelines published by the National Institute for Health and Care Excellence (NICE) advocate either Capecitabine monotherapy or FOLFOX for patients with stage III disease (NICE, 2011).

Approximately 30% of patients with stage II colorectal cancer develop recurrence and ultimately die of it. The case for giving all patients with stage II disease adjuvant chemotherapy remains controversial, given the associated toxicity (Benson, 2004). Guidance by NICE and ASCO suggests that offering adjuvant treatment to these patients is

at the discretion of the oncologist, taking into account the wishes of the patient (NICE, 2011; Benson, 2004).

1.6.4 Biological therapy

Biological therapies (targeted therapies) aim to modify the biology of the cancer cell. Important targets include the EGFR pathway (Cetuximab) or VEGF (Bevacizumab). These monocloncal antibodies are currently licenced for use in metastatic colorectal cancer only. FOLFIRI and Bevacizumab combination therapy was significantly associated with extended progression-free survival when compared with FOLFIRI and placebo (Hurwitz, 2004). Cetuximab is associated with improved survival when combined with FOLFIRI in patients with KRAS wild-type tumours (Van Custem, 2009). These findings have resulted in NICE guidelines advocating Cetuximab as a first line treatment in patients with isolated hepatic metastatic colorectal cancer and fit enough for surgery (NICE, 2011).

1.7 Inflammation and colorectal cancer

1.7.1 Introduction

Inflammation is now presented as a 7th 'hallmark' of cancer (Balkwill, 2001). Cancer-associated inflammatory responses can be divided into systemic (SIR) and local (LIR; within the tumour). Relationships between inflammation and survival depends on whether it is systemic or local (Klintrup, 2005; McMillan, 2008).

1.7.2 Local Inflammatory responses

Systemic inflammation is associated with a worse prognosis in colorectal cancer. In contrast, LIR appear generally protective. LIR represent complex multicellular and cytokine interaction with potential for tumour cell destruction. Evasion of this immunosurveillance is an important step in tumour metastasis (Watson, 2006). The LIR is composed of cells responsible for innate and adaptive immunity but mechanisms underlying cancer cell destruction by innate and adaptive mechanisms are still poorly understood

The benefit of lymphocytic infiltration has been known for a long time with McCarty and colleagues describing it as far back as 1931 (MacCarty, 1931). However, interest was renewed and has been sustained since Jass revisited it in 1986. Jass found that

in rectal cancer a pronounced inflammatory infiltrate predicted survival independently of depth of tumour invasion and lymph node metastasis (Jass, 1986). The LIR is variable in density and heterogeneous in terms of participating immune cells, with different patient survival outcomes. Delineating tumour and host characteristics that modify these responses may offer novel therapeutic targets.

1.7.2.1 The innate immune response

The immediate, innate immune response is not dependent of specific antibody or T-cell receptor recognition of antigens. It is largely implemented by dendritic cells, natural killer cells, neutrophils and macrophages, which kill pathogens by phagocytosis. Macrophages and dendritic cells process antigenic material derived from pathogens and present it to T-lymphocytes, establishing an adaptive immune response (Aderem, 2000; de Visser, 2006). The role of the innate immune response in colorectal cancer is controversial, with some evidence for effects on disease progression and anti-tumour activity. Translational studies of intra-tumour neutrophil infiltration have shown an adverse association with survival (Rao, 2012), but macrophages at the invasive edge predicted improved outcome (Forssell, 2007).

The acute inflammatory response plays an important role in tissue remodeling and angiogenesis and may even facilitate tumour escape from immune surveillance (Lin, 2007; figure 1.11).

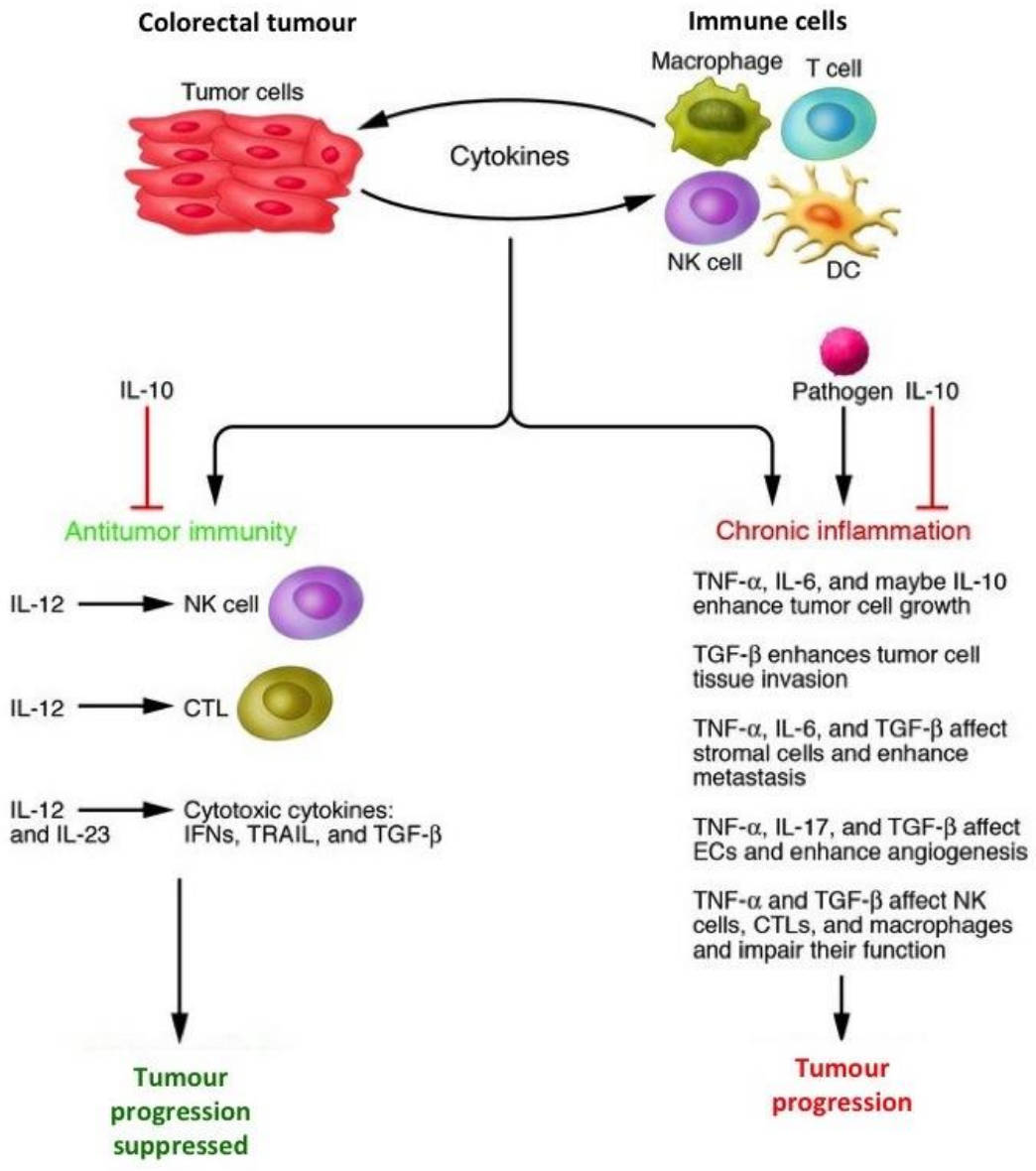


Figure 1.11: The role of the innate immune response in colorectal cancer progression and survival (*Adapted from Lin, 2007)*

1.7.2.2 The adaptive immune response

The adaptive immune response is a sophisticated multi-cellular and humoral response to antigen presentation by the innate immune system. Adaptive immune cells including $CD4^+$ helper lymphocytes and $CD8^+$ cytotoxic lymphocytes are antigen specific and result from expansion of specific clones recognition of foreign antigens. These clonal $CD8^+$ cells are created originally by randomly occurring rearrangements in a antigen specific-receptor and thus recognize diverse antigens.

Tumour-infiltrating lymphocytes are independently associated with improved survival (Naito, 1998). Naito and colleagues were the first to recognize that $CD8^+$

lymphocytes specifically within cancer-cell nests gave the most valuable prognostic information. It appears that cancer-cell nest $CD8^+$ infiltration represents a coordinated and specific anti-tumour immune response. The exact mechanisms underlying $CD8^+$ infiltration remains unclear but mismatch repair (MMR) deficient tumours have been associated with the presence of $CD8^+$ lymphocytes within the cancer cell nests (Dolcetti, 1999; Soreide, 2006).

1.7.3 Systemic inflammatory response

The systemic inflammatory response (SIR) protects the host against harmful agents by increasing vascular permeability, activating and stimulating proliferation of immune cells, initiating angiogenesis and promoting tissue remodeling (Coussens, 2002a). The SIR is a complex biosystem with multiple cellular and protein components. The cellular components include neutrophils, lymphocytes and platelets, with other immune cells present in smaller numbers. The protein components, collectively termed the acute phase protein response (APPR), are synthesised by the liver and help promote the inflammatory response. APPRs are defined as plasma proteins, which increase in concentration by greater than 25% in the first seven days following tissue injury (Kushner, 1982). Interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor (TNF) have been identified as the prime inducers of hepatic APPRs (Thompson, 1992).

1.7.3.1 Quantification of the systemic inflammatory response

The systemic inflammatory response has no specific endpoint and therefore developing an ideal biomarker is challenging. Several biomarkers have been proposed, most of which are based on differential serum white cell count ratios and APPR expression levels. Absolute values of the total white cell count, neutrophils and lymphocytes have long been used in assessing inflammation in patients, however more recently the ratio of circulating neutrophils to lymphocytes (Zahorec, 2001) has also been proposed. C-reactive protein (CRP), which is secreted by the liver in response to IL-6 (Bataille, 1992), is a reliable clinical marker of systemic inflammation and is commonly used to guide response to treatment in inflammatory conditions, along with the differential white cell count.

C-reactive protein

Two phases of response contribute to an elevated CRP; acute and chronic. CRP levels may increase acutely in response to trauma, infection, surgery and return to normal with

resolution of the causative factor. Secondly, CRP can be chronically raised and this has been associated with poorer survival in patients with metabolic syndrome and diabetes (Sattar, 2003).

In colorectal cancer many studies have looked at the role of CRP in predicting survival in patients with inoperable and operable colorectal cancer (Leitch, 2007; Roxburgh, 2010b, Roxburgh, 2010c). CRP offers stage independent prognostic information, but its effect on tumour cellular behavior remains unclear. Raised serum CRP has been associated with more rapidly proliferative tumours, but the pathophysiology underlying this remains unknown (Canna, 2008). It is likely that growth factors of the humoral systemic inflammatory response promote tumour progression via complex interactions with intracellular signaling pathways.

Albumin

The SIR is dependent upon specific amino acids for the generation of protein mediators such as immunoglobulins. This results in progressive loss of the albumin store and the development of hypoalbuminaemia (Fearon, 1998; Fearon, 1999; McMillan, 2001).

In colorectal cancer, hypoalbuminaemia has been strongly associated with CRP expression and poorer prognosis (Al-Shaiba, 2004; McMillan, 2001). The exact mechanism for this remains unclear as do relationships between hypoalbuminaemia and tumour characteristics.

1.7.3.2 The systemic inflammatory response in the context of colorectal cancer

Associations with poorer survival in both operable and inoperable colorectal cancer (Roxburgh, 2010b) implicate the SIR in tumour progression and metastasis. It is not clear how the prognostic value of the SIR relates to a model of tumour heterogeneity based on genomic instability. There is good evidence that MSI tumours are associated with a pronounced inflammatory infiltrate; however, it remains unknown if there is any association between systemic inflammation and MSI in colorectal cancer.

In non-cancer states, angiogenesis, tissue breakdown and remodeling are coordinated by the systemic inflammatory response. For example, in normal colon cell lines, IL-6 stimulates proliferation and inhibits apoptosis by increasing STAT3 expression via Janus kinase (Grivennikov, 2009). In cancer, studies examining relationships between the SIR and intracellular signaling pathways related to tumour behavior including invasion

are needed. It is likely that tumour progression and metastasis is based on a complex balance between tumour behaviour and the host.

1.8 Matrix Metalloproteinase 9 and cancer

1.8.1 Introduction

The matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases, first discovered in the 1960s, responsible for degradation of extracellular matrix (Gross, 1962). MMP-9, a gelatinase, is secreted as a 92-kDa proenzyme, and degrades type IV collagen following activation.

There are over 20 MMPs. They are classified by their location and early assessments of their substrate specificity as gelatinases, collagenases, stromelysins, and membrane-type MMPs. The gelatinases MMP-2 and MMP-9 degrade mainly type IV collagen. MMP-9's association with connective tissue degradation has implicated it in colorectal cancer progression and metastasis.

1.8.2 Structure of MMP-9

MMPs all share a common structure, with pro-peptide, catalytic and haemopexin-like C terminal domains. The catalytic and C-termnal domains are linked by a flexible hinge region. The pro-peptide region contains a conserved cysteine residue that interacts with a divalent zinc ion in the active site and prevents binding and cleavage of the substrate, keeping the enzyme in an inactive form. Within the catalytic domain a groove contains the zinc ion within the active site. Gelatinases such as MMP-9 incorporate fibronectin type II modules inserted immediately before the zinc-binding motif in the catalytic domain (figure 1.12). The haemopexin-like C-terminal domain contains a four-bladed β-propeller structure, which provides a large flat surface for protein interactions and determines substrate specificity. It is the site for interaction with tissue inhibitors of metalloproteinases (TIMP's).

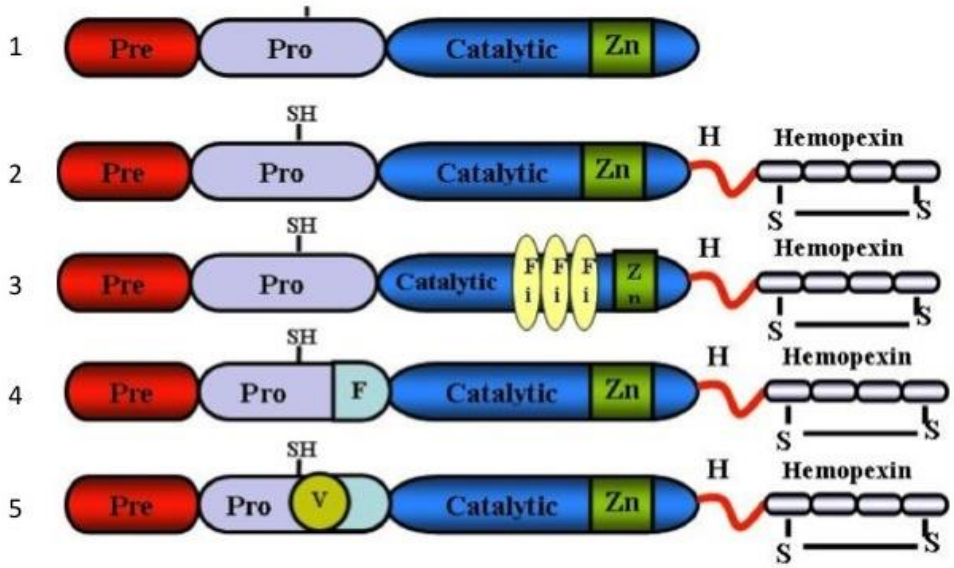


Figure 1.12: Structure of the matrix metalloproteinases (MMPs) (*Adapted from Di Carlo 2012)*

1. Minimal domain of MMPs, 2. Archetypical MMPs with simple hemopexin-domain, 3. Gelatin binding MMPs (MMP-9), 4. Furin activated MMPs, 5. Vitronectin insert MMPs. SH: thiol group, Zn: zinc binding, Fi:fibronectin, Fu:furin, Vn:vitronectin.

1.8.3 MMP-9 and colorectal cancer

The source of MMP-9 within colorectal cancers remains unclear. Tutton and colleagues found a close relationship between plasma concentrations and tumour expression of MMP-9 (Tutton, 2003). However, unlike tumour MMP-9, plasma MMP-9 was not associated with survival (Tutton, 2006). This finding appears paradoxical and a matched tumour and serum study is required. MMP-9 in the tumour is thought to originate from fibroblasts and immune cells in the microenvironment, (Roeb, 2001; Collins, 2001), however, MMP-9 mRNA has also been seen within the cancer cells (Koskensalo, 2012).

MMP-9 has been implicated in colorectal cancer development and progression through degradation of the basement membrane and other connective tissues of the colon including possibly in the walls of colonic veins (Zeng, 1995). MMP-9 expression was higher in tumours with synchronous liver metastasis (stage IV) compared to those with stage I-III disease (Matsuyama, 2002). Although this suggests that MMP-9 may plays a role in venous invasion, confirmatory evidence is unavailable. Given these associations with disease progression and metastasis, it is unsurprising that MMP-9 expression within the tumour has been associated with stage independent poorer survival (Zeng, 1996).

Colorectal adenocarcinomas are heterogeneous tumours with different molecular and biological characteristics based on well-defined genomic instability patterns. It is

possible, therefore, that aberrant MMP-9 expression is characteristic of an aggressive subgroup of colorectal adenocarcinoma with a specific molecular genetic pattern. Moran et al observed that MSI tumours were associated with lower levels of *active* MMP-9 despite higher levels of total MMP-9 (Morán, 2002). As well as moderate sample size (n=101), this study used tumour tissue that was not microdissected, and probably incorporated non-neoplastic tissue in the homogenate. MSI tumours are often heavily infiltrated by immune cells, which contain both inactive and active MMP-9, so these results need to be interpreted with caution. The association between MMP-9 expression and MSI status needs to be examined in a larger cohort using a histological model for quantification so that targeted areas of the tumour can be evaluated.

1.8.4 MMP-9 Inhibitors

Despite evidence implicating MMPs in tumour progression and survival, clinical trials of MMP inhibitors have been disappointing (Overall, 2002; Coussens, 2002b). A possible explanation is that the MMP-9 inhibitors used in these studies were either broad spectrum of only semi-selective, with higher dose-limiting toxicity and insufficient clinical benefit. Experimental MMP-9 specific inhibitors modify disease activity of ulcerative colitis and colorectal cancer in mouse models, but there are no mature trials in humans (Marshall, 2015). Another potential method for MMP-9 inhibition is via mechanisms that regulate expression of the molecule, such as the EGFR signaling (Westermarck, 1999), but further experimental evidence is needed prior to conducting clinical trials.

1.8.5 MMP-9 as a biomarker for treatment stratification

For MMP-9 to be useful as a biomarker in clinical practice, the techniques used to quantify it will probably need to discriminate between its active and inactive forms, while being reproducible and cost effective. Available antibodies to MMP-9 bind to an area common to the active and inactive forms, and do not allow one to discriminate between the activation status of the protein using antibody-mediated protein capture.

High expression of the protein at a particular cellular location may act as a surrogate marker of activation and this requires further study. Molecular weight quantification alone does not offer useful information regarding tumour cell specific protein expression as the homogenate will also include immune cells and nearby normal tissue. Therefore, developing a reliable and easily reproducible histological scoring method is needed but is likely be rather challenging. Serum MMP-9 has also been studied using

ELISA, however, relationships with tumour expression and whether it offers useful prognostic information require further study.

MMP-9 appears to offer useful prognostic information, based mainly on its relationship with tumour invasion. It is possible, however, that individual poor patient survival is attributable to other properties of the tumour. If MMP-9 is to be translated into clinical practice then it will need to be proven that it offers prognostic information independent of the clinicopathological factors currently used in clinical practice.

1.9 Src kinase family members and cancer

1.9.1 Introduction

The Src family kinases (SFKs) are non-receptor tyrosine kinases. They are regulatory proteins, with key roles in cell differentiation, motility, cell proliferation and survival (Frame, 2002). Src family kinases Blk, Fgr, Fyn, Hck, Lck, Lyn, c-Src, and Yes are all expressed in different human tissues.

1.9.2 Structure of Src

Src family kinases share a common protein structure (figure 1.13). The highly conserved Src homology domains, SH2–4, and a regulatory domain R, are common to all SFKs. SFKs are down-regulated by phosphorylation of a tyrosine residue at the 527 position, causing phosphorylated Tyr527 to interact with SH2 forming a "closed" conformation. In contrast, phosphorylation of a tyrosine residue at the 416 position results in the activation of SFKs.

SFK proteins are 60kDa tyrosine kinases with an N-terminal 14-carbon myristoyl sequence, a SH4 domain, SH3 and SH2 domains, a protein-tyrosine kinase domain, a short C-terminal regulatory tail and a unique segment (figure 1.12). N-terminal myristolation is required for membrane attachment and transforming activity by oncogenic Src mutants (Frame, 2002). The amino-terminal SH4 domain is unique to each SFK. The four distinct Src-homology domains (SH1-4) regulate SFK activity and interact with substrates to form intracellular signalling complexes (Ly, 2007). SFK activation is dependent on the interaction of different SH domains with each other and a C-terminal domain (Elsberger, 2009).

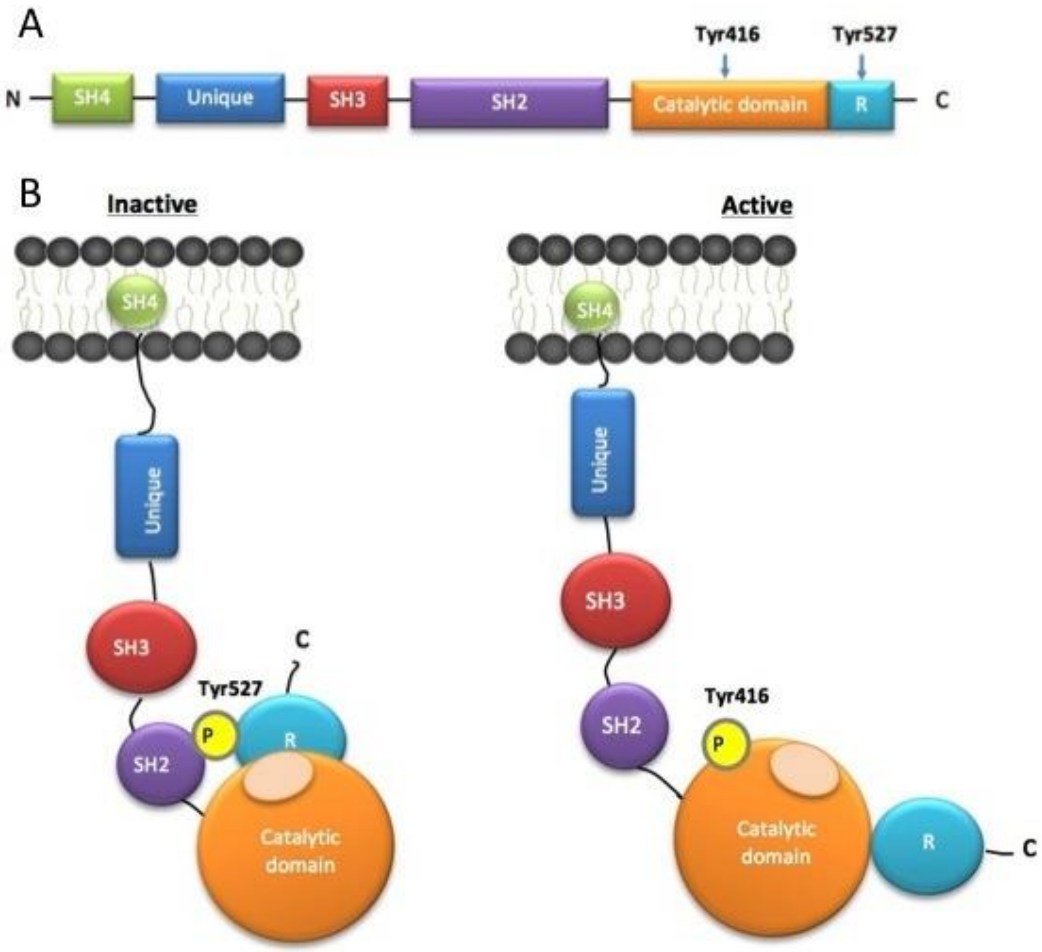


Figure 1.13: Structure of a Src family kinase (Adapted from Sicheri, 1997)

A.) The basic common structure of all SFKs. B.) Demonstrates the postulated change in structure between inactive and active forms of a generic SFK.

1.9.3 Activation of Src

SH domain interactions are highly dependent upon phosphorylation of various tyrosine residues within the protein. Inactive SFKs can be phosphorylated at the tyrosine 527 site by C-terminal Src kinase (ctSK) and ctSK homology kinase (Chk). Tyr527 is a highly conserved site among all SFKs located in the C-terminal tail. Mutations resulting in hypophosphorylation or loss of the C-terminal tail produce a constitutively activated protein. Phosphorylation of this site results in inactivation of the SFK by promoting binding of the C-terminus to the SH2 domain and binding of the SH3 to the SH1 domain (Cooper, 1993; Ly, 2007).

Physiological activation follows dephosphorylation of the Tyr527 site by tyrosine phosphatase and autophosphorylation of the tyrosine 416 active site (Roskoski, 2005).

Consequent unfolding of the protein allows substrate access to the protein kinase domain (figure 1.13 and Elsberger, 2009). Several substrates bind to the unfolded active areas. Growth factors such as EGFR and VEGFR, factors with their own kinase activities, can bind to the SH2 and SH3 domains, which can also bind to and activate cytoskeletal proteins such as FAK (Yeatman, 2004). It is thought that these interactions cause translocation of the SFK to their site of action (Elsberger, 2009).

Elsberger and colleagues note that SFKs are found at different cellular locations. These include, membrane, cytoplasm, perinuclear and the nucleus (Campbell, 2008; Elsberger, 2009). The SFKs are located mostly in the cytoplasm, however upon activation, they translocate to the cell membrane (Fincham, 2000).

1.9.4 Src kinases and colorectal cancer

There is little evidence about the role of Src and other SFKs in colorectal cancer progression and survival. c-Src is an independent predictor of poorer prognosis and its expression was higher in liver metastases than primary carcinomas (Aligayer, 2002; Han, 1996; Talamonti, 1993). On the other hand cell line studies have suggested that c-Src alone enhances tumour growth but not metastatic potential (Irby, 1997).

Selective silencing of c-Yes induced apoptosis and inhibited growth of HT29 colon carcinoma cell lines whilst inhibiting cell migration and metastatic capabilities in mouse models (Sancier, 2011). No translational studies have been done on the clinical significance of SFKs expression and activation in predicting survival in colorectal cancer.

Given the emergence of molecular subclassification of colorectal cancers, it remains unknown if SFK expression is associated with or indeed exclusive to a particular molecular ‘type’ of colorectal cancer. It is possible that aberrant SFK expression is associated with more aggressive subgroups of colorectal adenocarcinoma with a specific molecular genetic pattern.

1.9.5 Src kinase inhibitors

The high degree of homology between all SFKs has made it difficult to develop inhibitors to any particular family member. The commonly used Src inhibitors exert their effect on all family members. There are two main categories of Src inhibitor, the SH2/SH3 inhibitors and ATP-competitive kinase inhibitors.

1.9.5.1 SH2/SH3 inhibitors

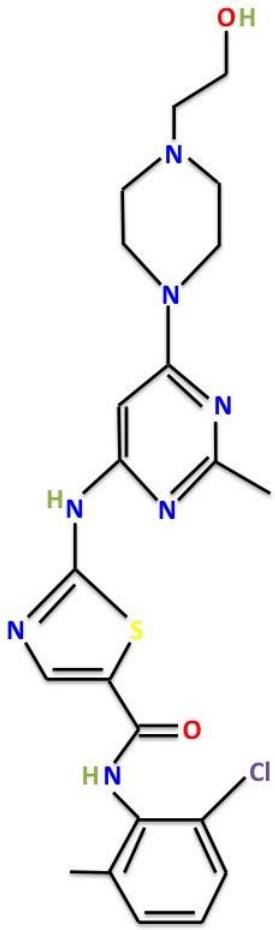
There are currently three principal SH2/SH3 inhibitors being studied; AP22408, and KX2 391, UCS15A. They antagonize Src by blocking its activation of downstream signaling substrates. AP22408 has osteoclast-targeting properties, with the potential to specifically target bone metastases while avoiding unwanted inhibition of Src-dependent activities in other cells types (Shakespeare, 2000). UCS15A interacts with the SH3 domain of the SFK and inhibits SH3-mediated protein-protein interactions in colorectal cancer cell lines (Oneyama, 2002). KX2 391 is an orally available, non-ATP– competitive Src inhibitor, which targets the substrate-binding ability of SFK. It appears to inhibit proliferation in hepatocellular cancer cell lines (Lau, 2009). Phase I trials have reported good tolerability, favouring phase II trials (Naing, 2013).

1.9.5.2 ATP-competitive Src kinase inhibitors

ATP-competitive SFK inhibitors work by inhibiting the kinase activity of the SH1 domain. There are currently three orally active inhibitors undergoing phase I or II clinical trials: Bosutinib (SKI-606, Wyeth), Dasatinib ((SPRYCEL, Bristol-Myers Squibb) and Saracatinib (AZD0530, AstraZeneca) (Daud, 2011; Kaye, 2012; Sharma, 2012).

Saracatinib and Bosutinib have anti-proliferative, anti-migratory and anti-invasive activity in several cell lines including breast, colon, prostate and lung cancer (Chang, 2008; Coluccia, 2006; Green, 2009; Hiscox, 2007). However, *in vivo* results are less conclusive with heterogeneity of results dependent on the underlying primary (Golas, 2005; Green, 2009; Jallal, 2007; Messersmith, 2009). Of the ATP-competitive inhibitors studied to date, Dasatinib has shown promising results both *in vitro* and *in vivo* and is in phase II trials. However, Dasatinib also targets other kinases, including bcr-abl.

Dasatininb has been licensed for the treatment of certain types of leukaemia (Brave, 2008). Multiple dosing regimens for Dasatinib have been proposed ranging from 50mg to 120mg once daily. This equated to peak blood concentrations of 41 ng/ml (50mg) and 85 ng/ml (100mg) (European Medicines Agency, 2006). Preclinical studies in colorectal cancer cell lines and colon cancer bearing mice have demonstrated reduced expression of activated SFKs and downstream markers of Src activation, with reduction in cell proliferation and integrin-dependent cell adhesion and migration (Serrels, 2006). In EGFR-dependent lung cancer cells, Dasatininb was found to selectively induce apoptosis (Song, 2006). Figure 1.14 below demonstrates the chemical structure of Dasatinib.



Molecular Formula: $C_{22}H_{26}ClN_70_2S$

Figure 1.14: The chemical structure of Dasatinib

1.9.6 SFKs as biomarkers for treatment stratification

Phase II trials of SFK inhibitors have not so far demonstrated clinical effectiveness (Sharma, 2012). Why remains unclear, but an important factor may relate to patient identification and the need for a predictive biomarker. The eight SFKs have all been associated with regulation of different physiological processes. One SFK inhibitor in particular, Dasatinib, blocks auto-phosphorylation of tyrosine 416 thus deactivating the family member. A predictive biomarker to identify patients who will benefit from Dasatinib therapy would be very useful.

Before SFKs can be used as prognostic or predictive biomarkers in clinical practice, reliable and cost effective methodology is needed to quantify their expression. Antibodies to individual SFKs bind to a common area on active and inactive forms. Antibodies used to identify SFK activation, via phosphorylation of Tyr416, target a phenomenon common to all family members and therefore there is currently no method of

discriminating between each activated member. A possible solution is that expression of the family member at a particular cellular location may act as a surrogate marker of SFK activation but confirmation is required. Clarifying these associations may or may not support a role for SFK expression in stratifying patients for Src inhibitor therapy.

1.10 Summary

Colorectal cancer is the third commonest cancer in the world with 31,000 new diagnoses each year in the UK alone. Five year survival is about 50% (Cancer Research UK, 2014). As in any illness, effective treatment requires accurate prognosis. Assessment of disease severity (prognosis) by pathological (TNM) staging is quite good (though capable of improvement), but prediction of responses to specific treatments is less successful. Surgery remains the mainstay of treatment, with curative or palliative intention. Despite undergoing potentially curative resection many patients will relapse. Reduced recurrence and better survival following surgery have been achieved by improved surgical technique and adjuvant oncological therapies.

Nevertheless, giving adjuvant chemotherapy to all patients undergoing potentially curative resection cannot be justified. Chemotherapy has significant morbidity and mortality and not all patients undergoing potentially curative resection need it. Therefore, identifying patients who do require and are likely to benefit from adjuvant therapy is clinically important. TNM staging is of use in stratifying patient risk. Patients with node positive carcinomas are substantially more likely to suffer cancer recurrence and these patients receive adjuvant therapy if fit. The clinical dilemma, however, particularly relates to people with node-negative cancers. Patients with early local disease (stage 1) have 95% 5 year survival, but stage II disease is much more unpredictable. The exact reasons for this are multifactorial and probably relate to the biology of the cancer as well as limitations of staging.

Histopathological assessment alone is insufficient in accurately stratifying patients for adjuvant treatments. Limitations of sampling can result in stage migration or under staging. No really reliable and reproducible grading system is available for colorectal cancer (Chandler, 2008). Although pathological features such as differentiation, necrosis, mucin production and serrated morphology have been associated with patient outcome, it is unlikely they can reliably be introduced into future TNM staging systems due to problems of reproducibility. Therefore, there is a prognostic gap which novel biological markers (biomarkers) might be able to bridge. In breast cancer, classical morphological

approaches are augmented by immunohistochemistry and *in situ* hybridization for assessment of oestrogen and progesterone receptors, and human epidermal growth factor receptor (Her2/*HER2*) expression/amplification. A current clinical challenge is to identify a biomarker or biomarkers that are both sufficiently predictive and prognostic to help improve treatment stratification for colorectal cancer patients.

In biomedical research, a biomarker is a measurable molecule that is a product of a normal or aberrant physiological process. Biomarkers are widely used in clinical medicine and may be diagnostic, prognostic or predictive. Diagnostic biomarkers tend to follow disease activity and have to be specific and sensitive to that condition. Prognostic biomarkers offer information on tumour aggressiveness and the likelihood of a poor outcome at diagnosis whereas predictive biomarkers are utilised to assess potential response to treatments.

Biomarkers to be introduced into clinical practice must undergo validation after being identified as candidates. The rigour of this process should ensure only genuinely useful biomarkers are introduced into clinical practice; many potential biomarkers fall short. Reasons for failure include insufficient cost benefit, inadequate reproducibility of the assay itself or its reporting. The invasiveness (onerousness) of the diagnostic test may also be a factor. With regards to cancer, it is currently unclear what is the best tissue to study. Historically tumour tissue itself has been the main focus of biomarker studies, but recently patient serum is increasingly studied. The advantage of serum is that obtaining it is less invasive and does not disrupt the tumour, which may influence disease progression. Despite this, tumour tissue from biopsies and resections remains the mainstay of prognostication.

Recent advances in the understanding of colorectal cancer suggest it is a heterogeneous disease with several genomic instability pathways including chromosomal instability, microsatellite instability, and epigenetic silencing of genes by promoter hypermethylation (Toyota, 1999). These pathways have associations with specific anatomical, histological and molecular biological behaviors, and variable outcomes in terms of recurrence. Tumours with MSI may be larger while still node negative, have a pronounced lymphocytic response and relatively better outcomes than their microsatellite stable counterparts (Soreide, 2006). Despite this, MSI tumours show chemoresistance to commonly used agents such as 5-FU (Guastadisegni, 2010; Hong, 2012). Given the complexity around survival parameters and predictive response to chemotherapeutic agents, its unclear how these might fit into a complex model of stratification based on the

TNM staging, but it seems likely that any future molecular biological staging process is likely to augment TNM staging, not replace it.

Tumour behavior is important and understanding it better may offer novel therapeutic options. New therapeutic targets may modify the behaviour of the tumour to make it less aggessive or more sensitive to chemotherapy. It is likely that future staging systems are going to integrate anatomical and biological aspects of disease.

Cancer related inflammation has been described as a 7^{th} hallmark of cancer (Balkwill, 2001). Tumours grow through a combination of dysregulation of cellular energetics, excess cell proliferation, loss of apoptosis, loss of cell adhesion and cell migration with tissue remodeling and dysregulation of angiogenesis (Hanahan, 2011). Inflammation plays an important part in all of these.

Despite systemic inflammation being associated with more aggressive disease, the presence of immune cells, particularly $CD8^+$ lymphocytes, in and around the advancing edge of the tumour has been associated with improved outcome (Naito, 1998). A complex relationship exists between the tumour and the host, which is both beneficial and detrimental. MSI tumours have been associated with a pronounced lymphocytic infiltrate within cancer-cell nests (Dolcetti, 1999), however, it remains unknown if there is any association between MSI tumours and the presence of systemic inflammation.

There is growing evidence that NSAID use reduces the transition from adenomatous polyp to cancer and improves outcome in established cancers (Garcia-Albeniz, 2011). Like other drugs, anti-inflammatories are unlikely to be appropriate for all patients and understanding the associations between the molecular biology of colorectal cancer and systemic inflammation may offer further useful information in stratifying patients for further treatment.

Cancer invasion requires breakdown of type IV and other collagens and extracellular matrix components, with increased tissue remodeling. Precise mechanisms in colorectal cancer are still unclear, but matrix metalloproteinase 9 (MMP-9) is a gelatinase that breaks down type IV collagen and may facilitate invasion and metastasis (Zeng, 1995). Active MMP-9 is present in colorectal cancers and is secreted by immune cells in the bowel wall, but it is unclear where else MMP-9 is produced and where it has most biomarker potential. MMP-9 has been reported in the tumour cells, the stroma and the serum; however, associations between these sites, general expression and prognosis remain unknown. Not all cancers express MMP-9 and it has not been adopted in clinical-pathological use. Why only some cancers express cellular MMP-9 remains unknown, however, it may relate to the molecular biology of the cancer. It has been suggested that CI

tumours are associated with higher expressions of MMP-9; however, this was a preliminary study using PCR, and validation using histological techniques is required (Morán, 2002).

Other cellular behaviours important for cancer progression are proliferation, apoptosis and cellular motility. Src kinase family members (SFKs) are non-receptor tyrosine kinases implicated in the biological processing of these behaviours. Inhibitors of SFKs activity have been studied, but phase III trials have not established a case for their clinical use (Sharma, 2012). Why remains unclear, but identification of suitable patients is likely to be important and a predictive biomarker might help. The eight SFKs have all been associated with regulation of different physiological processes. One SFK inhibitor in particular, Dasatinib, blocks auto-phosphorylation of tyrosine 416 thus effectively deactivating all family members. Most preliminary work has looked at the expression of c-Src in relation to adverse biological parameters and prognosis, but it is becoming clearer that other family members are also important.

Apart from cell line studies, associations between the expressions of the other SFKs have not been studied, and the effects of Dasatinib on the growth colorectal cancer cells and its effects on the cellular location of SFKs remain unknown. As for MMP-9, translational studies of SFKs suggest that their expression is heterogeneous and it remains unknown whether there is any association between genomic instability and expression of SFKs. Investigating these relationships and associations further may help with patient risk stratification and treatment through building a staging system based on anatomical, molecular and genetic cancer parameters.

1.11 Hypotheses and statement of aims

Colorectal cancers include some with MSI and a better prognosis, which has been widely investigated and may be due to local inflammation as a host defense mechanism against the tumour through cell destruction by the infiltrating immune cells. In contrast, systemic inflammation is associated with a poor outcome. MSI tumours are immunogenic, however, it is unknown if there is any association between MSI tumour status and systemic inflammation.

The mechanisms that drive the more aggressive phenotype resulting in the poorer prognosis associated with CI tumours is largely unknown. MMP-9 degrades the extra-cellular matrix and basement membrane, enhancing metastatic potential. Src kinase was the first identified proto-oncogene and increases tumour cell proliferation, decreases

apoptosis and increases the invasive capacity of the tumour. Therefore, an increase in the expression of Src family kinases and or MMP-9 could result in a more aggressive phenotype associated with CI tumours.

1.11.1 Core hypothesis

The core hypothesis is that increased expression of Src family kinases and MMP-9 is a feature of CI tumours and poorer outcomes. MSI tumours are associated with systemic inflammation but will not have a poorer outcome because of the positive effects of the local inflammatory response on patient survival.

1.11.2 Secondary hypotheses/aims

Secondary hypotheses/aims are:

1. The SIR will be associated with poor survival despite the association with MSI tumours.

2. Serum MMP-9 will be associated with stromal and cancer cell expression of MMP-9, and also associated with poor survival

3. On multivariable analysis of a cohort of patients with serum and matched tumour samples, cancer cell expression of MMP-9 will retain independent significance.

4. Dasatinib will not have a universal measured effect on proliferation and apoptosis across different cell lines.

5. Cellular location of the SFK proteins is an important predictor of cancer cell behavior and will change following treatment with Dasatinib.

6. When combining MSI, CRI, SFK and MMP-9 status/expressions into a multivariable model, cancer related inflammation will retain independent prognostic value in the whole cohort and TNM stage II colorectal cancer.

2. Materials and Methods

2.1 Patient Cohort selection

Patients with histologically proven colorectal cancer who, on the basis of pre-operative staging and findings at laparotomy, were considered to have undergone potentially curative resection for colorectal cancer were studied. Four cohorts were developed using patients treated for colorectal cancer from 1991 to 2012 across four hospital sites; Gartnavel General Hospital, Glasgow Royal Infirmary, Stobhill Hospital and Western Infirmary Glasgow and are detailed below.

Table 2.1: Details of the 4 cohorts

Cohort	Description	Years studied	Hospitals	Tissue type
1	Training	1997 - 2008	Glasgow Royal Infirmary	FFPE
2	Validation	2000 - 2008	Gartnavel General Hospital Stobhill Hospital Western Infirmary Glasgow	FFPE
3	Matched serum and tissue	2009 - 2012	Glasgow Royal Infirmary Gartnavel General Hospital	FFPE and Serum
4	Frozen tissue	1991 - 1996	Glasgow Royal Infirmary	Fresh frozen tissue

Development of the training and validation cohorts was undertaken by identifying patients either during discussions at the regional MDT or from retrospective review of the pathology specimen database, which included elective and emergency patients. Patients for cohort 3 ‘matched serum and tissue’ were recruited prior to surgery and serum samples collected on the day prior to elective surgery. The frozen tissue cohort was already available and formed the basis of the Department of Surgery, Glasgow Royal Infirmary Biobank.

Based on discussion at the MDT, patients were managed according to best practice guidelines available at the time of surgery. This consisted of either surgery alone, surgery with neoadjuvant therapy or surgery and adjuvant chemotherapy. Adjuvant therapy was given to patients at the discretion of the clinical oncologist, usually after multidisciplinary

team assessment. Patients' clinical and pathological information was available to the oncologist making these decisions. Ethical approval was obtained from the Glasgow Royal infirmary Local research Ethics Committee.

2.1.1 Sample collection

2.1.1.1 Tumour tissue

Resected samples were placed in formalin and following pathological dissection, representative areas were paraffin embedded as part of the NHS diagnostic pathology service.

2.1.1.2 Preparation and storage of blood samples

Blood samples were collected on the day before surgery and were prepared and analysed by the NHS biochemistry laboratory.

2.1.1.3 Preparation and storage of 'experimental' blood samples – cohort 3

Blood samples (n=125) were collected for analysis during diagnostic workup. This consisted of 95 patients and 30 controls. 10mls of whole blood was collected in EDTA tubes and immediately centrifuged at 2000 rpm for 15 minutes. The resulting cellular component and buffy coat was stored at 4^{o}C and the serum was aliquoted into new 1.5 ml Eppindorfs and stored at -80^{o}C.

2.1.2 Clinical characteristics

Laboratory measurements of albumin and C-reactive protein (CRP) concentrations prior to surgery were recorded. Preoperative measurements of albumin and CRP were dichotomised as described by McMillan et al (McMillan, 2007). Patients were considered to have an elevated CRP if measurements were >10 mg/l and a low albumin if measurements were <35 g/l. Demographic information including age and gender was also recorded. Patients were categorised as either elective or emergency based on the mode of presentation.

2.1.3 Pathological characteristics

Tumours were staged according to TNM 7th edition and the components of this staging system and tumour differentiation were recorded from pathology reports issued at the time of resection.

Tumour site was obtained from pathology reports; patients with tumours proximal to the splenic flexure were classified as right sided, those with tumours distal to and including the splenic flexure and proximal to the rectosigmoid junction were considered left sided and those distal to and including the rectosigmoid junction were considered rectal tumours.

Klintrup scoring was carried out as described according to a four-point score based on the appearances of tumour invasion at the deepest area (Klintrup, 2005). Score 0 indicated no increase in the inflammatory cells at the deepest point of the tumours invasive margin; a score of 1 denoted a mild and patchy increase in the inflammatory cells; a score of 2 denoted a prominent inflammatory reaction forming a band at the invasive margin with some destruction of cancer cell islands and a score of 3 denoted a florid 'cup-like' inflammatory infiltrate at the invasive edge with frequent destruction of cancer cell islands. These scores were then subsequently aggregated as low grade (scores 0 and 1) or high grade (scores 2 and 3).

2.1.4 Limitation of cohort studies

Cohort studies offer the best form of study conditions to allow us to predict the likely outcome of patients being treated for a particular disease over a certain timeframe, but reliability of the results is determined by how closely the study cohort represents the naturally occurring treatment group that is established during routine clinical practice. Furthermore, multiple cohorts from the same geographical area or overlapping of cohorts may result in 'overfitting' of data. Therefore, confirmatory findings in cohorts from diverse geographical areas are needed to control for this bias.

The method of recruitment to a cohort also influences the outcomes observed. Outcome measures of patients who present symptomatically are different from their screening counterparts beyond the stage shift seen at diagnosis. The lead time bias seen in screening programmes, whereby patients are diagnosed, staged and treated earlier than their symptomatic counterparts also affects outcome measures.

For cohort studies to be representative of the general disease specific population, adequate numbers of patients need to be recruited so that the sample has sufficient

statistical power. This may take several years and over this time routine practice may change, which affects survival measures, may result in stage shift and ultimately the results of the studied variables. Despite this, ensuring that all patients meet strict inclusion criteria and that a wide geographical area is studied reduces the risk of bias being introduced but does not eliminate it.

2.2 Immunohistochemistry

Immunohistochemistry detects of a cellular protein or other antigen within cells and tissues using an antibody specific for the desired antigen. The direct immunohistochemical method, when a marker is directly attached to the primary antibody, does not have a very high sensitivity. An alternative more sensitive method is indirect IHC. This involves using a second or "secondary" antibody, labeled with either a visible marker (fluorochrome) or an enzyme that binds to the primary antibody bound to the antigen. This indirect approach generates an amplified signal. Two methods of indirect IHC were used in this thesis, the Envision system (DAKO) and UltraVision Quanto system (Thermo-Scientific).

Dako Envision detection reagent consists of a dextran backbone to which many large number of horseradish peroxidase (HRP) molecules and secondary antibody molecules have been coupled. A unique chemistry is used for the coupling reaction, which permits the binding of up to 100 HRP molecules and up to 20 antibody molecules per backbone. The secondary antibody coupled to the dextran backbone has been raised in goats. It reacts equally well with rabbit and mouse immunoglobulins. Following incubation with the Envision reagent, the tissue is incubated with a substrate solution that consists of diaminobenzidine (DAB) chromagen and hydrogen peroxide. The HRP molecules on the Envision interact with the substrate solution to produce an insoluable brown end product at the site of the target antigen/protein, which can be viewed using a light microscope.

The Thermo-Scientific UltraVision Quanto detection reagent consists of a universal secondary antibody formulation that is conjugated to an enzyme-labeled polymer. This conjugate also reacts equally well with rabbit and mouse immunoglobulins. Following incubation with HRP Quanto, the tissue is then incubated with a substrate solution consisting of DAB chromagen, exactly as with the DAKO Envision system.

2.2.1 Stages of the immunohistochemistry technique

2.2.1.1 Tissue preparation

IHC was performed on 4 μm, archival formalin-fixed, paraffin-embedded colorectal tumour sections. Sections were dewaxed in xylene (2x5 minutes) and rehydrated through graded alcohols (100% (2x3minutes), 90% (1x3minutes) and 70% (1x3minutes)) washes.

2.2.1.2 Antigen Retrieval

After formalin fixation and paraffin embedding of tissues, many antibodies do not react with their antigen. Solvents, heat and especially fixatives can mask the antigen site. During routine fixation, methylene bridges form, which cross link proteins and mask the antigenic sites. Therefore it is usually necessary to include an antigen retrieval step, to break the protein cross-links and expose the antigenic binding site. This reduces non-specific background staining by allowing reduced concentrations of antibody to be used in the primary incubation.

Two different heat mediated methods of antigen retrieval were used for the proteins studied. The first involved incubating the tissue sections under pressure in 1L of TE buffer (1 mM EDTA (Sigma), 10 mM Tris (VWR), pH 9.0) or 1L of Citrate buffer (10 mM of citric Acid, 10 mM Sodium Citrate, pH 6.0) for 5 minutes. The alternative method incubated tissue sections for twenty minutes at 96°C in a PT module using Thermo-Scientific HIER antigen retrieval solution (low: pH 6.0 and high pH 9.0). All antigen retrieval steps were followed by a twenty-minute cool down period.

2.2.1.3 Reduction of background staining: - Blocking steps

Peroxidase reacts with DAB and endogenous peroxidase activity in tissues can cause background staining. We blocked endogenous peroxidase activity by incubating the slides in 3% hydrogen peroxide (H_2O_2) or H_20_2 Quanto for ten minutes. The formation of hydrophobic bonds between immunoglobulins and tissue proteins can lead to the primary and secondary antibodies binding non-specifically to the tissue section rather than the target antigen. To reduce this non-specific binding, tissue sections were incubated in either 2.5% normal horse serum (Vector Laboratories) in TBS buffer (0.1M Tris/HCl, 1.5M NaCl, pH 7.4) for twenty minutes, Casein (DAKO) for one hour or Ultra V block for 5 minutes.

2.2.1.4 Incubation with Primary Antibody

Antibody dilutions, incubation times and temperature were established for each protein investigated on control section of colorectal cancer. All antibodies were diluted to the desired concentration in antibody diluent (DAKO or Thermo-Scientific). For each, a dilution series was performed, investigating various antibody titrations, incubation times and temperatures, to establish optimal conditions for the highest quality of staining, i.e. strongest specific antigen staining with the lowest non-specific background. Positive and negative controls were included each time IHC was performed. The positive control confirmed that the chosen IHC method was working while the negative control checked that the detection system was not generating a spurious signal on its own. Tonsil tissue, placenta tissue or colorectal tissues previously shown to have strong expression of the desired antigen were incubated with the appropriate antibody and used as positive controls. The same tissues were used for negative controls by treating them with a negative isotype matched control reagent (DAKO).

2.2.1.5 Incubation with Secondary Antibody

Following incubation with antibody or negative control, the slides were thoroughly washed in TBS buffer twice for five minutes. The Envision detection or UltraVision method was used for all antigens. The slides were incubated with Envision for thirty minutes then washed twice for five minutes in TBS or HRP Quanto for 5 minutes followed by a TBS wash step.

2.2.1.6 Detection & Visualisation

The chromagen used for staining the tissue sections was 3,3'-diaminobenzidine (DAB) – (5ml distilled water (dH_2O), 2 drops of buffer solution, 4 drops of DAB stock solution, and 2 drops of Hydrogen Peroxidase solution -Vector Laboratories) or DAB Quanto (Thermo-Scientific). Slides were incubated with DAB for five to ten minutes to allow brown staining to develop and were then washed in running water for ten minutes.

2.2.1.7 Counterstaining

Tissue sections were counterstained with haematoxylin and Scott's Tap Water Substitute (S.T.W.S). Slides were immersed in the haematoxylin for 2 minutes, until a red colour was produced in the tissue section. Following this, slides were then submerged in S.T.W.S for

90 seconds, to produce a blue counterstain in contrast to the brown positive staining of the antigen. The last steps involved dehydrating the tissues through a series of alcohol washes: (70% (1x2min), 90% (1x2min), 100% (2x2min)) and xylene (2x2min), and then mounting the slides onto coverslips using DPX mountant (VWR) (Dibutyl Phtalate containing Xylene).

2.2.2 Tissue Microarray construction (Cohorts 1 and 2)

Prior to beginning TMA construction, the author underwent a period of tuition on GI tract histology with a consultant pathologist. This included the recognition of normal tissue and colorectal neoplasia with particular focus on identifying tumour rich areas and discerning between viable and necrotic tissue. Miss Clare Orange and Dr Jonathan Platt constructed the TMA for cohort 1.

2.2.2.1 Slide retrieval, identification of tumour areas and slide marking

Following identification of patients to be included in the study, pathology numbers were used as unique patient identifiers. H&E slides of the representative areas of tumour were collected for every patient (7-8 slides per patient). Every section was reviewed under the microscope, marking areas of cancer. Given that tumours are heterogeneous in cohesion and differentiation, multiple areas of the section were marked. A consultant pathologist reviewed a proportion of the marked slides agreeing with the selection of normal and neoplastic areas. Patients with no H&E sections available were removed from the study.

2.2.2.2 Creation of TMA maps

Factors such as the size and number of cores needed, minimum distance between cores, rows and columns and availability of control tissue were all taken into account when planning TMAs. The training cohort TMA was already available and has a different layout to the validation cohort TMA.

The TMA for cohort 1 contains four 0.6 mm diameter cores lined adjacently into three columns: 4 cores in the first column, 8 cores in the second and 8 cores in the third. One core of six control tissues (Rectal cancer, Lung, Liver, Cervix, Kidney, Prostate) was included on each TMA. 40 cores of representative areas of normal tissue were also included. Appendix 1.1 demonstrates the plan for cohort 1 (training cohort) TMA.

To minimize possible bias, which may have been introduced by having all cores from one patient adjacent to each other, a different plan was implemented for cohort 2. Four 0.6 mm diameter cores of tissue were planned from each patient with only one core on each individual TMA block. This process was replicated four times so that tissue from a single patient was spaced across four TMA blocks. Single cores of six control tissues (rectal cancer, lung, liver, cervix, kidney and prostate) were included on each TMA. A 1mm distance was placed between each core with regular larger gaps of 2mm between columns. A map was then created using a spreadsheet with all the above factors taken into account. Sixteen TMAs were needed to include the 759 patients, however, only 677 patients were included in the final study. Each core area is given a unique ID to allow linkage analysis with TMA database whilst maintaining patient anonymity. Appendix 1.2 demonstrates the plan for the validation cohort TMA.

To facilitate optimization of antibodies and avoid unnecessary use of full TMA tissue sections, a practice TMA containing 48 cores of colorectal cancer was also designed.

2.2.2.3 TMA construction

Following marking of the relevant H&E sections, all the corresponding FFPE blocks were retrieved from the tissue archive at Stobhill Hospital. All FFPE tissue blocks were then transferred to the pathology department at the Southern General Hospital. The author then underwent a dedicated period of training at the SGH into the manufacturing of TMAs under the auspices of Ms Clare Orange, TMA Manager, University Department of Pathology. A manual tissue arrayer (Beecher Instruments microarray technology) was used to create a number of practice lines of tissue cores from different pathological specimens. Once the desired standard of TMA construction was met, the author went on to create all practice and full TMA's. Below is a step by step guide for TMA construction:

1. Four blank wax blocks are fixed into position within the magnetic turntable.
2. A core of wax from a pre-planned position in the recipient block is removed using the red needle.
3. A core of tissue, from an area pre marked on the matched H&E section, is removed using the blue needle.
4. The donor core of tissue is placed gently into the recipient hole. Using a glass slide, the remainder of the tissue is gently introduced into the block. The tissue core is slightly larger than the recipient hole, which allows a tight fit.
5. This process is repeated on all four blank blocks by rotating the magnetic turntable.

6. Using the micrometer, the coring mechanism is moved along the block until the next desired location is reached. Steps 2-6 are then repeated until the TMA is complete.
7. Following completion of the TMA, each block is placed in a pre-heated oven at 60^{o}C for 5 minutes; this allows the cores to set within the block. Once completed, each block is wrapped in a paper towel and stored in a cool dry place.

2.2.3 Immunohistochemistry on the training cohort TMAs

Immunohistochemistry was performed on 4 μm TMA sections using the DAKO Envision methodology described in section 2.2. All sections were baked at 60^{o}C for 30 minutes prior to dewaxing to improve adherence of cores to the slides. Dewaxing and rehydration of sections was followed by heat induced antigen retrieval. Sections were incubated in 3% H_2O_2 for 30 minutes and then protein binding blocker. Sections were washed in TBS and then incubated with antibody followed by further washes and signal amplification by DAKO envision for 30 minutes. Sections were then washed, incubated in DAB chromogen for 10 minutes followed by further washes and counterstaining in haematoxylin. Finally, sections were dehydrated and mounted with DPX and cover slipped. Immunohistochemistry conditions for each antibody are given in table 2.2.

Table 2.2: Immunohistochemistry antibody details for cohorts 1 and 3.

Protein	Company	Antigen Retrieval	H_20_2	Blocker	Dilution	Incubation	Amplifier
MMP-9	Millipore	None	0.3%	1.5% HS	1:75	30 min	Envision
Src416	Cell Signaling	pH 9.0	0.3%	1.5% HS	1:50	ON	Envision
FAK861	Invitrogen	pH 6.0	0.3%	1.5 %HS	1:100	ON	Envision
HCK	Cell Signalling	pH 6.0	0.3%	5% HS	1:50	ON	Envision
FGR	Abgent	pH 9.0	0.3%	Casein	1:2000	60 min	Envision
FYN	Millipore	pH 6.0	0.3%	5% HS	1:2000	ON	Envision
LCK	Cell Signaling	pH 9.0	0.3%	5% HS	1:100	ON	Envision
MLH1	Thermo Fisher	pH 9.0	Peroxidase block	UV Block	1:100	20 min	Quantro
MSH2	Thermo Fisher	pH 9.0	Peroxidase block	UV Block	1:50	20 min	Quantro
MSH6	Thermo Fisher	pH 9.0	Peroxidase block	UV Block	1:100	20 min	Quantro
PMS2	Thermo Fisher	pH 9.0	Peroxidase block	UV Block	1:50	20 min	Quantro

2.2.4 Immunohistochemistry on cohorts 3 full section tissue

Immunohistochemistry was performed on 4 μm full sections using the DAKO Envision methodology described in section 2.2. Dewaxing and rehydration of sections was followed by heat induced antigen retrieval. Sections were incubated in 3% H_2O_2 for 30 minutes and then protein binding blocker. Sections were washed in TBS and then incubated with antibody followed by further washes and signal amplification by DAKO envision for 30 minutes. Sections were then washed, incubated in DAB chromogen for 10 minutes followed by further washes and counterstaining in haematoxylin. Finally, sections were dehydrated and mounted with DPX and cover slipped. Immunohistochemistry conditions for each antibody are the same as discussed in section 2.2.3.

2.2.5 Immunohistochemistry on the Validation Cohort TMAs

Immunohistochemistry was performed on 4 μm TMA sections using the ThermoScientific Ultravision methodology using a ThermoFisher autostainer. All sections were baked at 60^{o}C for 30 minutes prior to use. Sides were placed in a PT module whereby dewaxing, rehydration and antigen retrieval was performed automatically. This consisted of warming sections to 90^{o}C, incubating at this temperature for 20 minutes and then a further 20 minute cool down. Sections were then transferred to the autostainer. Sections were incubated in UV H_2O_2 for 30 minutes and then UV protein blocker. Sections were washed in TBS and then incubated with antibody followed by further washes and signal amplification by UltraVision for 30 minutes. Sections were then washed, incubated in UV DAB chromogen for 10 minutes followed by further washes and counterstaining in haematoxylin. Finally, sections were dehydrated and mounted with DPX and cover slipped. Immunohistochemistry conditions for each antibody are given in table 2.3

Table 2.3: Immunohistochemistry antibody details for cohort 2.

Protein	Company	Antigen Retrieval	Endogenous blocker	Blocker	Dilution	Incubation	Amplifier
MMP-9	Millipore	None	Peroxidase block	UV Block	1:50	20 min	Quantro
FAK861	Invitrogen	pH 6.0	Peroxidase block	UV Block	1:100	20 min	Quantro
HCK	Cell Signalling	pH 6.0	Peroxidase block	UV Block	1:50	20 min	Quantro
FGR	Abgent	pH 9.0	Peroxidase block	UV Block	1:400	20 min	Quantro
MLH1	Thermo Fisher	pH 9.0	Peroxidase block	UV Block	1:100	20 min	Quantro
MSH2	Thermo Fisher	pH 9.0	Peroxidase block	UV Block	1:50	20 min	Quantro
MSH6	Thermo Fisher	pH 9.0	Peroxidase block	UV Block	1:100	20 min	Quantro
PMS2	Thermo Fisher	pH 9.0	Peroxidase block	UV Block	1:50	20 min	Quantro

2.2.6 Immunohistochemistry quantification

2.2.6.1 Histoscore method

The 'semi quantitative' histoscore method was used to assess protein expression at the nuclear, cytoplasm and membrane locations (MacCarty, 1986). This method of scoring has been validated previously and is widely used including by our team and others. The intensity of staining was categorized as negative (0), weak (1), moderate (2) and strong (3). The percentage of tumour cells with each category was estimated. The histoscore of the tumour areas was calculated using the following formula:

Histoscore = (0 x % negative cells) + (1 x % weakly stained cells) + (2 x % moderately stained cells) + (3 x % strong stained cells)

The histoscore ranges from a minimum of 0 to a maximum of 300 and was calculated independently for nucleus, cytoplasm and cell membrane. Agreement between two

observers was considered good if ICCCs were greater than 0.7 and excellent if greater than 0.8.

2.2.6.2 Assessment of mismatch repair protein expression

Mismatch repair protein (MLH1, MSH2, PMS2 and MSH6) expression was evaluated using UK NEQAS scoring guidelines. Appendix and normal colon were used as positive controls. Appropriate control staining determined by (i) strong nuclear expression in immune cells, (ii) strong nuclear expression in the base and lower half of the normal crypts with fading of intensity near the top of the crypt adjacent to the luminal surface and (iii) strong nuclear staining in lymphoid follicles. An observer blinded to genomic MSI data and clinical outcome scored 10% of cores.

Expression was reported as normal (strong nuclear staining with positive immune cells), patchy/weak (staining intensity is either weak or patchy with retention of normal immune cell staining) or negative (complete loss of expression with normal immune cell expression). Perinuclear immunopositivity alone was not considered diagnostic for protein expression. Patchy staining in the cytoplasm with normal immune cell expression was considered to be result of MMR protein complex destabilisation with loss of binding to the nuclear DNA (Arends, 2008).

2.2.7 Limitations of immunohistochemistry

2.2.7.1 Immunohistochemistry techniques

Immunohistochemistry is often used for the detection and quantification of biomarkers because it is relatively simple to perform, offers useful information on the cellular location of the protein and is less expensive than other techniques. Despite this, it has limitations that need to be considered when deciding if it is appropriate for protein identification and quantification within a particular study. Although technically not challenging, optimisation of immunohistochemistry requires experience to ensure appropriate specificity of the test. Broadly, the limitations of immunohistochemistry can be divided into reaction bias and interpretation bias.

Reaction bias is a result of issues surrounding tissue fixation, processing, antigen retrieval and detection. During fixation, formalin causes methylene bridges to form. This cross-linking around the antibody binding site on the protein reduces its immunoreactivity. Prolonged and especially variable fixation therefore, can result in variable

immunoreactivity between and within cases, even using identical antigen retrieval conditions. The aim of antigen retrieval is to recover immunoreactivity of the recognition site of the protein to increase the sensitivity of the antibody being employed. Using lower concentrations of the primary antibody has the added benefit of improving specificity. Variations in antigen retrieval methods and secondary detection system result in apparently different protein expression within the same tumour sample. The rigor of the immunohistochemical techniques employed within diagnostic pathology is assessed by international quality programs such as UK NEGAS. In addition, journals commonly request authors to report the results of their biomarker studies using guidelines such as the REMARK criteria. This measure attempts to control for bias by ensuring that authors use previously validated methodology, which also offers the added benefit of wider validity of interpretation of the results within the field of study.

Interpretation bias includes issues surrounding antibody selection, sensitivity/specificity and results interpretation. A poor primary antibody could have a poor sensitivity, by not binding to the desired protein, and poor specificity by binding to other proteins. In an attempt to identify issues surrounding antibody sensitivity and specificity antibody panels undergo a process of validation. Western blot bands will help identify the specificity of the antibody, which can also be supported by performing the immunohistochemistry reaction with a blocking peptide. Another useful technique is to confirm loss of signal in cell lines that have been treated with siRNA to reduce expression. It is also necessary to observe the immunoreaction pattern of the antibody panel in negative and positive controls as well as internal and external controls. These validation studies themselves are also associated with limitations and thus all immunohistochemical reactions need to be interpreted with caution.

2.2.7.2 Immunohistochemistry quantification - 'scoring'

Immunohistochemistry is relatively cheap, easy to perform, reproducible and methodologically transferable. Despite this, there are very few instances where immunohistochemistry as a process for biomarker quantification has translated into routine clinical practice. One of the main reasons for this is the limitations surrounding reporting and interpretation of immunoreactivity, and especially in genuine signal quantification.

Various methods of quantification have been devised, however, these aim to generate an ordinal variable for a visual continuum. Scoring methods can range from positive and negative, ordinal or semi-quantitative such as the weighted histoscore as

discussed above. Despite all these efforts, any scoring method incorporating intensity and volume of immunoreactivity will be at best semi-quantitative and open to reporting bias. Another problem is that immunohistochemistry involves very high-gain signal amplification and small variations in gain can have a large effect on the final signal.

Quantifying the intensity of an immunoreaction also relies on the assumption that the chromagen follows the Beer-Lambert law that describes the linear relationship between the concentration of the molecule and its optical density. In immunohistochemistry, the immunoreactivity is not stoichiometric and in particular the chromagen DAB is not a true absorber of light and thus dark and light stains have different spectral shapes. Therefore, it is not possible to precisely measure how much of the molecule is present using optical density alone.

2.3 ELISA

2.3.1 MMP-9 ELISA

The Human MMP-9 Quantikine ELISA kit (R&D Systems, UK) was used to assess MMP-9 expression in the serum of colorectal cancer patients. This assay is a solid phase Sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) and begins with a monoclonal antibody specific for MMP-9 coated onto the wells of microtitre strips. Samples, including a standard containing MMP-9, control and plasma samples, are added to these wells. During the first incubation, the protein antigen binds to the immobilized (capture) antibody. After washing away unbound substances, an enzyme-linked polyclonal antibody specific for MMP-9 is added to the wells. Following a wash to remove unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of MMP-9 bound in the initial step. The color development is stopped and the intensity of the color is measured. The intensity of this coloured product is directly proportional to the concentration of total or phosphorylated protein present in the original specimen.

ELISA was performed according to the manufacturer's instructions. The protein standards were prepared by reconstituting the standard 1 (20 ng of lyophilized of recombinant human pro-MMP-9) with 1ml of standard diluent buffer. To ensure complete reconstitution, the preparation was left at room temperature for ten minutes, on a stirrer. The remainder of the standards was then set up as follows:

Standard	Add	Into
20 ng/ml	1 ml of calibrator diluent buffer	20 ng of lyophilized of recombinant human pro-MMP-9
10 ng/ml	500 µl of 20 ng/ml standard	500 µl of calibrator diluent
5 ng/ml	500 µl of 10 ng/ml standard	500 µl of calibrator diluent
2.5 ng/ml	500 µl of 5 ng/ml standard	500 µl of calibrator diluent
1.25 ng/ml	500 µl of 2.5 ng/ml standard	500 µl of calibrator diluent
0.625 ng/ml	500 µl of 1.25 ng/ml standard	500 µl of calibrator diluent
0.312 ng/ml	500 µl of 0.625 ng/ml standard	500 µl of calibrator diluent

All sample reactions were performed in triplicate with the final value derived from the mean. Firstly, 100 µl of the assay diluent was added to each well of the 96 wells plate. 100 µl of standard was added to the first three columns of the plate with A1-3 having 20 ng/ml of MMP-9, G1-3 having 0.312 ng/ml and diluent only in H1-3. Plasma samples were then diluted 1:40 as per manufacturers guidelines. Negative controls consisted of wells containing 100 µl of diluent only. 100 µl of sample were then added the appropriate wells, covered with a plate sealer, wrapped in foil and incubated for 2 hours on a horizontal orbital microplate shaker.

The solution was then carefully removed from the wells and washed four times with washing buffer. 200 µl of conjugate solution was added into each well, covered with a plate sealer, wrapped in foil and incubated for 1 hour on a horizontal orbital microplate shaker. The solution was carefully removed and wells washed four times with washing buffer. Following this, 200 µl of substrate solution (combination of colour reagent A and B) was added to each well, covered with a plate sealer, wrapped in foil and incubated for thirty minutes at room temperature on the benchtop. 50 µl of stop solution was added to each well and the side of the plate tapped gently to ensure thorough mixing and even distribution of the protein for accurate concentration reading. This solution changes the colour form blue to yellow.

The absorbance was measured using a 96 well microplate reader at 450 nm having blanked the reader against a chromagen blank composed of conjugate solution and stop solution. Using Excel the absorbance of the standard against the standard concentration was plotted. The values obtained for the samples were multiplied by the dilution factor (40) to correct for the dilution.

2.3.1.1 Normalisation procedure for inter-plate variation

To allow for inter-plate variation, two samples of normal control plasma were included in each ELISA experiment. The correction factor was calculated by dividing the value of the control sample on each plate by the mean. Each patient sample was then multiplied by the correction factor for that plate. Apart from a control sample, each plate consisted of both normal controls and test samples chosen at random.

2.3.2 Limitations of ELISA

For quantifying potential biomarkers, ELISA has the benefit of being able to examine numerous samples simultaneously, whilst also being relatively easy to perform and identifies antigens at low concentrations. ELISA is therefore commonly used in clinical practice to measure serum markers to help diagnose and manage different conditions. Despite these positive features, ELISA is also associated with several limitations that mean results of biomarker experiments have to be interpreted with caution. One limitation of ELISA is the associated inter- and intra-assay variance. Methods to control for this include standards, a pre-defined positive control that is included on each plate and calibration.

Only monoclonal antibodies can be used and therefore assays can only detect a single target. Given that antigens may have multiple epitopes, ELISA is unable to distinguish between antigenically identical analytes, which increases the risk of false positive results. Furthermore, reliable monoclonal antibodies are relatively difficult to produce and therefore, ELISA may not be available for all potential biomarkers.

Interpretation of the immune reaction that underpins the quantification method for ELISA relies on measuring the optical density of the reaction using a spectrophotometric microplate reader. The enzyme/substrate immunoreaction is short term so 96 well plates must be read as soon as possible after the stop solution is applied. A delay in this process may result in ambiguous results.

2.4 In- vitro studies

2.4.1 Cell line choice

Matched metastatic T84 (lung metastasis) and non-metastatic HT29 (colon confined) colorectal cancer cell lines were purchased from ATCC, (UK). The T84 cell line was derived from a lung metastasis of 72 year old man with colon cancer, whereas the HT29 cell line was from a primary colorectal cancer of 44 year old woman.

Clinical trials examining the effect of novel biological therapies on outcome are largely based on stage IV colorectal cancer. It remains unclear if the response seen in primary disease mirrors that of metastatic disease. Therefore, both metastatic and primary cell lines were examined in this thesis.

2.4.2 Culturing of colorectal cancer cell lines

T84 cells were routinely maintained in DMEM: F-12 Medium (ATCC, UK) supplemented with 5% foetal calf serum (Invitrogen, UK), 2mM L-glutamine (Invitrogen, UK), and penicillin/streptomycin (50 units/ml, 50 μg/ml (Invitrogen, UK)). HT29 cells were routinely cultured in McCoy's 5A Medium (ATCC, UK) supplemented with 10% foetal calf serum (Invitrogen, UK), 2mM L-glutamine (Invitrogen, UK), and penicillin/streptomycin (50 units/ml, 50 μg/ml (Invitrogen, UK). Cells were grown in T-75 flasks (Gibco) and maintained in 5% CO_2 at 37^{o}C, with the medium changed twice weekly, as it is rapidly acidified.

2.4.3 Trypsinisation of cells

Sub-confluent cultures (70-80%) were routinely passaged 1:6 using trypsin (Invitrogen, UK) to prevent the cells becoming confluent and forming clumps. Used medium was removed from the flasks and the cells washed twice with warmed Phospho-Buffered Saline (PBS) (Invitrogen) to eliminate traces of serum, which includes trypsin inhibitors. Cells were then incubated in 3 mls of trypsin for 5 minutes in 5% CO_2, 37°C, in order to detach the cells from the flask. Once cells were no longer adherent, 3mls of representative culture medium was added to inactivate the trypsin. The cells were disaggregated from their clusters by gentle pipetting and seeded into new T-75 flasks containing 10 mls of fresh representative culture medium.

2.4.4 Freezing cells

Once cells are trypsinised, aliquots of the cells can be stored for future use. The cell suspension was transferred from the flask to a 15 ml centrifuge tube and cell pellets were collected by centrifugation at 1200 rpm for 5 minutes. The medium was removed and the pellet resuspended in 1ml of representative culture medium (supplemented 10% foetal calf serum (Invitrogen, UK), 2mM L-glutamine (Invitrogen, UK), and penicillin/streptomycin (50 units/ml, 50 μg/ml (Invitrogen, UK)) and 10% DMSO which serves as a

cyroprotectant. The cells were immediately transferred in an alcohol bath (Mr Frosty, Sigma) to -80^{o}C for 24 hours before being transferred to liquid nitrogen (-180^{o}C) for long-term storage.

When cell aliquots were required, they were removed from liquid nitrogen and warmed for 1 minute in a 37^{o}C water bath before being promptly transferred to a flask containing 10 mls of pre-warmed RPMI. It was essential not to leave the cells defrosting longer than necessary, as DMSO is toxic.

2.4.5 Drug treatments

2.4.5.1 Drug preparation

Dasatinib was stored at -20^{o}C with a stock concentration of 10 mM. T84 and HT29 cell lines were treated with control media (standard growth media only), vehicle media (standard growth media and DMSO) and drug media (Dasatinib in standard growth media). Dasatinib was prepared at 1 nM, 10 nM, 20 nM, 50 nM and 100 nM and 200 nM from the stock concentration of 10mM using serial dilutions. Given the results of pharmacokinetic studies, a dosing regiment of 50mg to 100mg twice daily would result in blood peak concentrations of between 42 ng/ml and 84 ng/ml (European Medicines Agency, 2006). Given that the molecular weight of Dasatinib is 488 g/mol a blood concentration of 100 ng/ml would equate to a drug concentration of 200 nM for *in-vitro* studies.

2.4.5.2 Cell line treatment in 96 wells plate

T84 and HT29 cells were seeded in 96 well plates at a density of 5000 cells per well (100 µl) in standard culture medium and grown until 60% confluent. Cells were then incubated in serum free media overnight. Cells were treated with either 100 µl of control, vehicle or drug media at the concentrations described in section 2.4.5.1 for a period of 24 hours, 48 hours and 72 hours. Each time frame experiment was performed in triplicate, on three separate occasions (n=9 for 24h, 48h and 72h; total of n=27).

2.4.5.3 Metabolic activity assay

Metabolic activity (cell viability) was assessed using the WST-1 (Water Soluble Tetrazolium Salts) assay (Millipore, UK). The cleavage of the tetrazolium salt WST-1 (water soluble tetrazolium salt, in the presence of 1-methoxy PMS) to formazan by cellular mitochondrial dehydrogenases represents mitochondrial and therefore cellular metabolic

activity. Expansion of viable cell numbers results in an increase in the activity of the mitochondrial dehydrogenases within the sample. This increase in mitochondrial dehydrogenase results in an increase in formazan dye metabolism. The formazan dye produced by the viable cells is measured at an absorbance of 440 nm using a standard multiwell spectrophotometer.

Cells were seeded in 96 well plates at a density 5000 cells (100 µl) per well in standard culture media. The assay was performed at 24, 48 and 72 hours by adding 10 µl of WST-1 reagent prior diluted in Electro Coupling Solution (ECS) to each well. The optical absorbance level was measured after 2 hours incubation at 37^{o}C, (this time point was determined in previous studies performed within our group) using a 96 well microplate reader at 450 nm with reference wavelength 600 nm. Each experiment was repeated three times and each condition was done in triplicate, thus the experiment was conducted 9 times.

2.4.5.4 Apoptosis assay

The Cell Death Detection ELISA Kit (Roche, USA) was used to detect apoptosis in both colorectal cancer cell lines treated with increasing concentrations of Dasatinib, diluted in culture media for 24 h, 48 h and 72 h. This is a one step sandwich ELISA for relative quantification of histone-complexed DNA fragments (mono- and oligonucleosomes) from the cytoplasm of cells after the induction of apoptosis. T84 and HT29 CR cells were seeded 5000 cells (100 µl) per well and cultured for 24 hours followed by incubation for either 24, 48 or 72 hours with 1 nM, 10 nM, 25 nM, 50 nM, 100 nM and 200 nM of Dasatinib, diluted in culture media. After the incubation, the cells were pelleted by centrifugation at 200 xg for 10 minutes at room temperature and the supernatant was discarded. The cells were then resuspended with 100 µl of lysis buffer and incubated for thirty minutes at room temperature. After lysis, the cells were collected by centrifugation at 200 xg and 20 µl of the supernatant was transferred to a streptavadin coated microtiter plate. 100 µl of immunoreagent (two monoclonal antibodies, antihistone (biotin-labeled) and anti-DNA (peroxidase- conjugated) was added to the wells and incubated at room temperature for two hours. The immunoreagent was carefully removed and wells washed three times with washing buffer to remove cell components that were not immunoreactive. Following this samples were incubated with peroxidase substrate for fifteen minutes at room temperature and absorbance of the samples was measured using a 96 well microplate reader at 405 nm.

2.4.6 Cell pellet studies

2.4.6.1 Cell line treatment with Dasatinib

Cell lines were grown in T-75 flasks until 60% confluent, the medium was removed and the cells washed in warmed PBS. Then, cells were incubated in serum free media overnight. The following day, medium was removed and the cells washed in warmed PBS and incubated with 50 nM of Dasatinib for 48 hours. A control experiment of just media was also included. The experiment was performed in triplicate.

2.4.6.2 Cell pellet formation

After incubation with drug media for 48 hours, the solution was removed and cells were carefully washed twice with warm PBS. 3 ml of trypsin was added to the flasks and incubated for 5 minutes at 37^{o}C in 5% CO_2 atmosphere. Trypsin was neutralised with the equivalent amount of media. The 6 ml suspension was pipetted gently up and down to dislodge all cells and transferred into a 15 ml centrifuge tube (Fisher) and centrifuged at 10,000 rpm for 10 minutes. This was followed by the supernatant being discarded and 3 mls of media being gently placed on top of the pellet. Each centrifuge tube was placed on ice until the experiment is completed.

The supernatant is then carefully discarded leaving a cell button at the bottom of the universal container. 2-3 drops of fridge stored human plasma is put on top of the cell button, combined with the cell pellet using a plastic pipette (Alpha laboratories) and subsequently mixed by moderate shaking of the container. Afterwards 1-2 drops of thrombin working solution is applied and gently agitated to allow a clot to form. Formalin is added slowly to avoid fragmentation of the clot. Fixation of the clot in formalin occurs overnight before being placed into a correctly labeled (drug media (D) and control media (C)) paraffin block cassette. The pellet was then and taken to histopathology for imbedding in a paraffin wax block.

2.4.6.3 Immunohistochemistry of cell pellets

Immunohistochemistry was performed on 4 μm full sections using the DAKO Envision methodology described in section 2.2. This consisted of dewaxing and rehydrating sections followed by heat induced antigen retrieval for only 2.5 minutes. Sections were incubated in 3% H_2O_2 for 30 minutes and then protein binding blocker. Sections were washed in TBS and then incubated with antibody followed by further washes and signal amplification by

DAKO envision for 30 minutes. Sections were then washed, incubated in DAB chromogen for 10 minutes followed by further washes and counterstaining in haematoxylin. Finally, sections were dehydrated and mounted with DPX and cover slipped. Immunohistochemistry conditions for each antibody is given in table 2.2

2.5 PCR studies

Quantitative real-time PCR (qRT-PCR) is a sensitive technique that uses oligonucleotide primers, dNTPs and Taqman polymerase to amplify DNA. A reverse-transcription step prior to qRT-PCR enables quantification of mRNA. Therefore, qRT-PCR is a commonly used method for quantifying gene expression.

The qRT-PCR reaction assumes a quantitative relationship between the amount of starting target sample and the amount of PCR product at any given cycle number. The reaction relies on a DNA polymerase with 5' exo-nuclease activity of which there are several commercially available systems such as TaqMan® system. The other key component to this reaction is a specific oligonucleotide probe, for a DNA sequence between the forward and reverse primers. These probes are designed with a high-energy dye at the 5' end termed a reporter and a lower energy dye at the 3' end termed a quencher. When the probe is intact the quencher suppresses the reporter dye emission. When the probe is cleaved by the 5' exo-nuclease, the energy from the reporter molecule is released and sensed by a fluorescence sequence detection system. Thus with each cycle of the PCR reaction there is an increase in the fluorescence emission detected by the reporter dye. The reaction is specific at three levels, the complementary probe cleaved during the amplification reaction along with the forward and reverse primers.

Quantification of gene expression using qRT-PCR requires normalisation of the reporter dye emission signal using a passive reference dye, which is incorporated into the PCR mastermix. The normalised reporter (Rn) value is quantified as the emission of the reporter dye divided by the emission of the normalised reporter. The ΔRn is defined as the change in Rn between an untreated or early cycle sample (Rn-, no template control) and a sample containing a full complement of reaction components including the target (Rn+). The ΔRn reflects the increase in signal, which indicates the amount of hybridised probe that has been degraded by the exo-nuclease. The Rn is used by the software of the detection system to define a baseline of fluorescence and a threshold, which is set in the exponential phase of the reaction. The threshold is calculated as the average standard deviation of Rn for the early PCR cycles. Finally, the threshold cycle (Ct) is the cycle

number at which fluorescence passes the fixed threshold. The Ct value is used in the final calculation of gene expression (Figure 2.2).

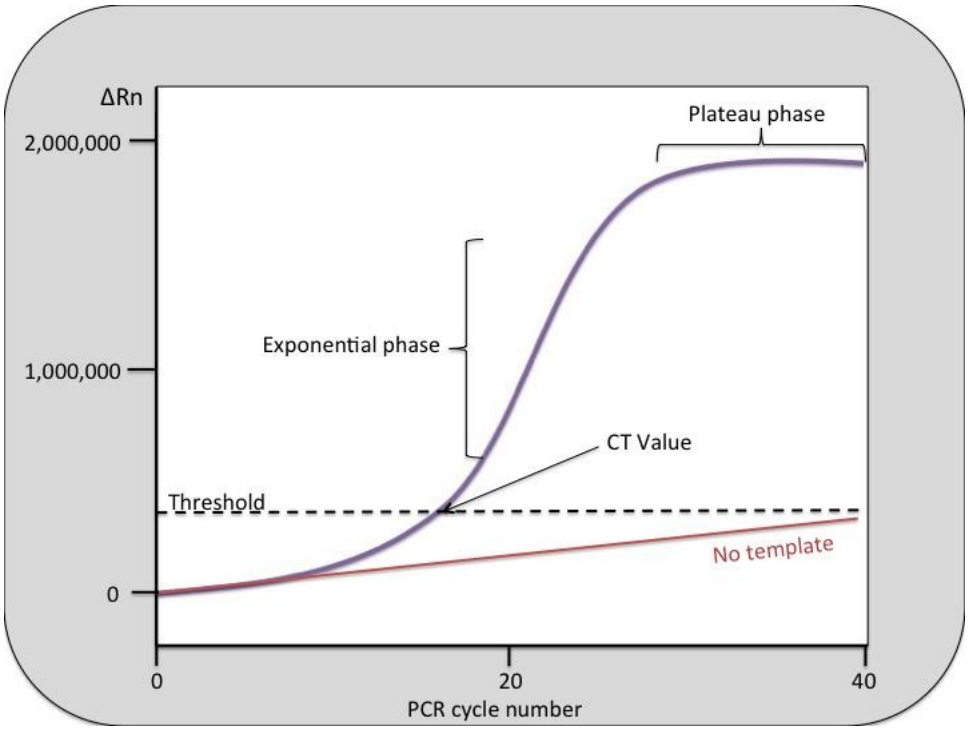


Figure 2.1: Example diagram of a real time qPCR amplification plot

2.5.1 Src kinase family members

Reverse-transcriptase qRT-PCR was used to determine mRNA expression of Src family kinases in colorectal tumours, which were snap frozen in liquid nitrogen at the time of resection.

2.5.1.1 RNA Isolation

50-75 mg of colorectal tumour tissue was homogenised in 1ml of TRIZOL® in a glass tube using an OmniTip plastic homogenizer probe. Once the tissue was completely homogenized, the homogenate was transferred to a clean Eppendorf tube and incubated at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes. 200 µl of chloroform was added to the homogenate, which was shaken vigorously for 15 seconds and a cloudy red solution developed. Samples were then incubated at room temperature for 3 minutes. Following this, samples were centrifuged at 13,000 rpm for 15 minutes at 4^{o}C. Using a large tip and avoiding dislodging the precipitate, the aqueous supernatant was removed and placed in an autoclaved Eppendorf

tube. 500 µl of isopropanol is added to the sample to precipitate the RNA and is incubated at room temperature for 10 minutes. The sample is the centrifuged at 13,000 rpm for 10 minutes at 4^{o}C. Following this, the isopropanol is carefully decanted to leave a small white pellet at the bottom of the tube. 1 ml of 75% ethanol is added the sample and mixed using a vortex.

To solubilize the RNA, the sample was centrifuged at 10,000 rpm for 5 minutes at 4^{o}C. The ethanol was carefully decanted not to dislodge the pellet which was left to dry for 10 minutes at room temperature. 30 µl of DEPC treated water was added to the sample. The sample was then mixed using a vortex, spun down and incubated at 65^{o}C on a heating block for 5 minutes. The sample was then quickly mixed, spun down and placed on the heating block for an additional 5 minutes. Once heating was complete the Eppendorf containing the sample was placed on ice. The RNA within the sample was quantified and the 260/280 ratio checked using a Nanodrop.

2.5.1.2 cDNA synthesis by reverse transcription

Prior to reverse transcription, the RNA sample must be DNase treated to reduce the possibility of DNA contamination. This is done by first diluting 5 µg of RNA in water to final volume of 21.5 µl. 2.5 µl of 10 x Dnase1 buffer and 1 µl of rDnase1 are then added to the dilution and spun down. The sample is then incubated at 37°C for 30 minutes. To remove the DNase Inactivation Reagent, it is first resuspended by vortexing the tube and 3 µl of slurry is added to the sample. Mixing is achieved by flicking the tube and the contents incubated for 2 minutes at room temperature. Following this, the sample is centrifuged at 13,000 rpm for 1 minute to produce a pellet of DNase Inactivation Reagent. The supernatant solution containing the RNA is then transferred to a fresh tube.

Reverse transcription of mRNA to cDNA was achieved using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, UK). 2x RT mastermix was prepared per sample as described in Table 2.4. cDNA synthesis was performed by adding 5 µl of 2x RT mastermix to 5 µl of DNase treated RNA sample. The samples was then spun down and incubated at 25^{o}C for 10 minutes, 37^{o}C for 120 minutes and then 85^{o}C for 5 seconds. The resulting cDNA was stored at -20^{o}C. When calculating cDNA, the equivalent RNA was used as cDNA has too many nucleotides to measure using the Nanodrop.

Table 2.4: Components of the 2x RT mastermix including a no RT control for a single sample

Component	Volume	
	RT	No RT control
10 X RT Buffer	1 µl	1 µl
25 X dNTPs	0.4 µl	0.4 µl
10 X random primers	1 µl	1 µl
Multiscribe reverse transcriptase	0.5 µl	0 µl
Superasin (1 U/ul)	0.5 µl	0.5 µl
Nuclease free water	1.6 µl	2.1 µl

2.5.1.3 Quantification of mRNA

qRT-PCR was performed using an ABI Prism 7900 Sequence Detection System (Applied Biosystems, UK) and TaqMan® Gene Expression Assays (Table 2.5). The PCR reaction mixture consisted of 12.5 µl of 2x Taqman mastermix, 1.25 µl of 20x target assay mix, 10.25 µl of DDW and 1 µl of cDNA. The manufacturer‘s protocol recommended 40 rounds of amplification. Thermal cycler condition were 50°C for 2 min, 95°C for 10 min followed by 40x 95°C for 15 sec and 60°C for 1 min. Product melting curve analysis and gel electrophoresis experiments were used to ensure that only one product of the expected size was amplified.

Negative controls (RNAse/DNAse free H2O and negative qRT-PCR sample) for each primer were included on every 96 well PCR plate (Applied Biosystems, UK). Quantitative values were obtained from the threshold cycle value at which the increase TaqMan® probe fluorescent signal associated with an exponential increase of each individual PCR product reaching a fixed threshold value. Each individual primer had a fixed threshold Ct value. These fixed threshold values were used for every cDNA sample.

To enable comparison of different mRNA expression levels, their relation to the average expression level of two housekeeping genes GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and HPRT (hypoxanthine-guanine phosphoribosyl-transferase) were evaluated. The housekeeping gene with the lowest standard deviation (HPRT) was used for evaluation of the different mRNA expression levels. Data were analysed using the Sequence Detection Software, which calculates the Ct value. The expression of the target assay was normalised by subtracting the Ct value of the housekeeping gene from the Ct

value of the relevant target assay. The fold increase, relative to the control, was obtained by using the formula 2-ΔCt, and then expressed as a percentage (x100).

Formula: 2 -(Mean Ct target gene- Mean Ct house keeping gene) x100

All samples were measured in triplicate. Supportive technical assistance was given by Fiona Jordan, laboratory technician, however, all experimental work and analysis was performed by the author.

Table 2.5: Primers used for SFK qRT-PCR reactions and their fixed threshold Ct values.

Gene	Gene expression Assay ID	Reporter	Exon boundary spanned according to product insert	Amplicon Length	Threshold value (Ct)
SRC	Hs01082246_m1	FAM	7-8	70	0.25000
LCK	Hs00178427_m1	FAM	9-10	104	0.20000
LYN	Hs00176719_m1	FAM	12-13	70	0.20000
YES	Hs00736972_m1	FAM	2-3	153	0.20000
FYN	Hs00941600_m1	FAM	3-4	99	0.20000
FGR	Hs00178340_m1	FAM	5-6	61	0.20000
HCK	Hs00176654_m1	FAM	6-7	64	0.20000
BLK	Hs01017452_m1	FAM	1-2	85	0.20000
GAPDH	4310884E	VIC	3	118	0.16014
HPRT	4310890E	VIC	6-7	100	0.17000

2.5.2 Microsatellite Instability

2.5.2.1 Slide retrieval, block identification and tissue preparation

H&E slides of the representative areas of tumour were collected for every patient. Every section was reviewed under the microscope and an estimate of overall tumour areas quantified as a percentage. This estimate of cancer nuclear material calculated against the background of normal epithelium, muscle, fat and immune cell infiltrate. For successful identification of microsatellite instability, 2 x 10 μm sections of tumour rich (>30%) FFPE tissue was needed for DNA extraction and PCR analysis.

To reduce the possibility of false negatives, tumours that had less than 40% tumour were macrodissected to an equivalent of 2 x 10 μm sections. This often resulted in dissection of 4-8 x 10 μm sections. During tumour quantification, H+E sections were assessed for ease of dissection and marked to allow identification of tumour areas at a later date. Representative blocks were chosen based on ease of dissection whilst keeping tissue wastage to minimum. During the dissection process, 10micron section were cut and placed

onto a non-adhesive glass slides. Using the pre-marked H+E, tumour areas were identified and dissected using an 11 scalpel blade. The dissected tissue was then placed in pre-labeled sample tube. Using a printed label with a barcode, each patient was given a unique identifier that was placed on the sample tube and accompanying elution tube.

2.5.2.2 DNA extraction

DNA was extracted from FFPE tissue by digesting the tissue prepared in section 2.5.2.1 in Qiagen ATL buffer containing proteinase K for 2 hours followed by extraction on the Qiagen Symphony using a QIAsyphony DNA kit (Qiagen, UK). Using a Nanodrop, the extracted DNA was quantified and the 260/280 ratios checked to ensure appropriate quality.

2.5.2.3 MSI Multiplex PCR analysis

PCR for MSI was performed using a G-Strom PCR thermocycler and Qiagen MSI primer sets (Qiagen, UK) in a multiplex reaction. Multiplex fluorescent PCR analysis was performed on 5 loci (BAT-25, BAT-26, NR-21, BR-24 and MONO-27, Table 2.x) that are routinely used in the NHS reference laboratory (Ninewells Hospital, Dundee) where this study took place. The PCR reaction mixture for a single sample consisted of 12 µl of 2x Qiagen Multiplex PCR kit, 3.5 µl of sterile water and the primer volumes described in Table 2.6. 24 µl of MSI multiplex reaction mixture and 1 µl of sample DNA was added to each well of a 96 wells plate and transferred to the G-Storm thermocycler. Thermo cycler conditions consisted of 95^{o}C for 10 minutes followed by 34 cycles of 94^{o}C for 1 minutes, 58^{o}C for 1 minute and 72^{o}C for a minute with a final step of 10 minutes at 72^{o}C.

Table 2.6: Markers, primers and volume used in the MSI multiplex PCR reaction mixture

Marker	Primer sequence	Volume in multiplex
NR21F-Hex	gagtcgctggcacagttcta	0.75 µl
NR21R	ctggtcactcgcgtttacaa	0.75 µl
NR24F-HEX	gctgaattttacctccgac	0.75 µl
NR24R	attgtgccattgcattccaa	0.75 µl
BAT26F-NED	tgactacttttgacttcagcc	0.5 µl
BAT26R	aaccattcaacatttttaaccc	0.5 µl
BAT25F-FAM	tcgcctccaagaatgtaagt	1.0 µl
BAT25R	tctgcattttaactatggctc	1.0 µl
MONO27F-FAM	gtggagattgcagtgagctg	1.25 µl
MONO27R	ggyggatcaaatttcacttgg	1.25 µl

Prior to setting up the genescan, PCR products were diluted 1:100 by aliquoting 99 µl of molecular grade water and 1 µl of PCR product into a new 96-wells plate. Using yet another new 96 wells plate, 9 µl of ROX/formamide aliquot (0.5 µl of GS500 ROX and 8.5 µl of deionized formamide) was placed into each well followed by 1 µl of diluted PCR product sample. The plate was then run on an ABI 3130 analyser and the results analysed using Genemarker software.

2.5.2.4 MSI analysis

MSI is defined as any length change due to either insertion or deletion of a repeating unit, in a microsatellite amplified from a tumour. Using recognised reporting guidelines by the local health board, patients were categorised as MSI-positive or CI based on the degree of instability:

MSI-H = Instability at 2 or more loci

CI = Evidence of instability at 1 or less loci studied

Technical assistance was given by Ms Christine Black who also taught the author how to perform the technique and analyse the results.

2.5.3 Limitations of PCR techniques

As a screening technique for studying many tumour samples, PCR has the limitations of being relatively expensive, difficult to perform and time consuming, although improvements in technology have alleviated the latter issue to some extent. Given these limitations, PCR is commonly used following a preliminary screening process such as immunohistochemistry in diagnosing Lynch syndrome, or strict clinical criterion such as screening for hypertrophic cardiomyopathy.

Even at low tumour DNA concentrations, PCR offers a robust method of identifying the presence of microsatellite repeats and has therefore been established as the gold standard test for diagnosing MSI. Quantitative PCR offers a relatively accurate representation of how much of a protein is likely to be present by indirectly measuring this through the number of mRNA copies present. Limitations in this process such as incomplete conversion of mRNA to cDNA does raise the possibility of erroneous results, even when controlling for other variable factors.

The quantification process of PCR assumes that the volume of tumour and specifically cancer cell DNA is identical in all samples. Colorectal cancer cells often have variable growth patterns as described by Jass (Jass, 1986). Thus the 'pushing' tumours have a higher concentration of cancer cells compared to the their infiltrative group. In addition, the presence of other non-cancer cells, such as the local inflammatory infiltrate, may 'dilute' the representation of the tumour DNA in the sample. Therefore, a specific weighted sample of tumour may have variable amount of tumour DNA present, which raises the possibility of erroneous results.

2.6 Biomarker identification and quantification - Rationale for choice of scientific techniques

All scientific techniques have strength and weaknesses when attempting to translate the quantification of a molecule into useful clinical information. The ideal biomarker quantification technique will be accurate, cost effective, easy to perform, non-invasive and specific to the tumour. Unfortunately, this hard to achieve and therefore multiple techniques and different sample types are commonly needed.

Allowing for the limitations of immunohistochemistry, it is relatively easy to perform, cost effective and offers useful information beyond simply representing how much is present. Because it is performed on FFPE sections, information regarding the cellular location of the protein, the characteristics of the immunoreaction as well as its

relationship with the local microenvironment is also available. The information obtained from full sections allows us to examine the relationships between the protein expression at multiple cellular locations and clinical parameters.

Quantitative PCR offers a useful and reliable yet indirect method of protein quantification as it measures preliminary substrates of an end product. It is relatively expensive to perform; time consuming and the final results represent the combined DNA of normal and cancer tissues. Although there are measures to control for this such as micro dissection, these are also time consuming with elevated costs. Given the need for frozen tissue to perform quantitative PCR, we have elected to use immunohistochemistry for protein quantification in the training, validation and matched serum cohorts. The false negative rate for MSI identification using PCR remains low as long as the cancer DNA proportion is at least 20% and therefore we have opted to perform the multiplex PCR method described above.

A relative limitation of immunohistochemistry and quantitative PCR is the need for tumour samples, which are obtained through invasive medical procedures. ELISA can be performed on patient blood samples, which is obtained through a less invasive method. ELISA is routinely used in clinical practice because it is relatively easy to use and large numbers of samples can be performed in a single experiment. Although it does not offer cancer specific information, it is a relatively good method of quantifying the presence of a protein despite its relative limitations.

2.7 Statistical methodology

Hypothesis testing is a statistical method of choosing between the null hypothesis and an alternative hypothesis. The falsification theory of Karl Popper suggests that we can never conclusively prove a hypothesis; we can only disprove it and thus we test the *null* hypothesis (Popper, 1959). Therefore, the absence of evidence does not mean the evidence of absence. To test the hypotheses proposed in this thesis, the data obtained will undergo a range of descriptive and inferential statistical tests. An assumption of inferential statistical tests is that the observed data is representative of a larger population and thus a range of tests are required to demonstrate the proportionality and adequacy/power of the sample.

2.7.1 Sample size calculations in biomarker studies

The adequacy of the sample size is vitally important in determining the validity of the test results. It is important to reduce the chance of incorrect interpretation of statistical tests by including a sufficient number of patients. The basis of the sample size calculation relies on numerous statistical and clinical factors and these are discussed below.

2.7.1.1 Statistical hypotheses testing errors

Type 1 error

Irrespective of the inferential statistical test employed, the probability of this observation occurring by chance alone needs to be taken into consideration. It is common practice to calculate a p-value which when <0.05 infers that the probability of this observation occurring by chance alone is below 5%. A type 1 error occurs when the null hypothesis is incorrectly rejected when in fact no association truly exists. The risk of a type 1 error is commonly described as α (alpha) and may take any value (0.05, 0.001 or <0.001) depending on the risk accepted by the researcher, however, $\alpha < 0.05$ is commonly used in the scientific literature.

Type 2 error

A type 2 error occurs when the null hypothesis is not rejected despite a true association existing. The risk of a type 2 error is described as β (beta) and the probability of not having a type 2 error is calculated as $1 - \beta$. Power in terms of statistical hypothesis tests relates to the probability of not having a type 2 error and is represented as $1 - \beta$. Adequate power, which is commonly set at >0.80, strengthens the interpretation and validity of the results. Allowing for weaknesses in scientific methodologies, the main confounding factor for a type 2 error is an inadequate sample size.

2.7.1.2 Sample size considerations

The issue of sample size determination in biomarker cohort studies remains controversial with many published studies appearing significantly underpowered on post-hoc evaluation (Altman, 2012). The sample size has to be large enough that the probabilities of type 1 and type 2 errors are acceptably low. Too large a sample size, however, is associated with heightened research costs due to excess tissue assessment. The recruitment of too many patients to a study could be considered unethical (mainly because it wastes resources) and

therefore a balance must be struck between adequate sample size and participant dropout due to compliance issues with tissue and follow-up data.

The power of survival tests ultimately relate to the number of endpoint *events* observed during follow-up. Therefore in biomarker studies, the sample size considerations relates to two broad areas, the interrelationships between studied variables and the relationship between studied variables and survival. Unlike data association tests, time to event outcome requires the events to have occurred rather than the sample size. Therefore, groups with higher event rates such as those with stage IV colorectal cancer will require fewer patients than with stage I colorectal cancer. This is particularly important if the study is going to stratify survival results by a particular clinicopathological factor.

Simulation studies have demonstrated that at least 10 events are required for each of the covariates considered (Bradburn, 2003). Multivariable models, which adjust the magnitude of the association between the studied variables and time to endpoint outcome based on confounding covariates, require more events per studied variable. Too few events will lead to unreliable results and loss of validity. About 25 events per variable has been proposed as desirable (Altman, 2012).

2.7.1.3 Sample size calculation methods

Sample size calculations for biomarker studies are generally power based as they relate to hypothesis testing. The statistical methodology for sample size calculation differs depending on whether the data is quantitative or categorical. Sample size calculations for hypothesis tests are heavily affected by the choice of α and β with lower values for both resulting in larger numbers for n when compared with the widely accepted values $\alpha = 0.05$ and $\beta = 0.2$.

Quantitative data

In order to determine the sample size required to compare the mean between two groups information on the α, β, $f(\alpha, \beta)$, δ (the smallest difference in the means) and s (the standard deviation of the measured variable).

$$n = f(\alpha, \beta) \times (2s^2 / \delta^2)$$

Categorical data

The sample size calculation for categorical data is based on the expected proportion of observed events in a test group, which would be present if there was a clinically significant difference between the two groups. This method of calculating sample size ensures that the study is adequately powered to observe the clinically relevant minimum effect size. The sample size calculation is calculated using the formula below

p1 = proportion of events in group 1

p2 = proportion of events in group 2

α = significance level

β = type 2 error probability

$$n = (p_1 (1 - p_1) + p_2 (1-p_2) / (p_1 - p_2)^2) \text{ x } f(\alpha,\beta)$$

2.7.1.4 Limitations in the application of power calculation

The main limitations for sample size calculations in biomarker studies relate to the choice of values for α and β by the researcher. These values relate to the probability of observing type 1 or type 2 errors respectively. Although the widely accepted type 2 error rate is 20%, this still equates to the incorrect non-rejection of the null hypothesis in twenty occasions out of one hundred inferential tests. Another limitation is the lack of widely accepted sample size calculations for time to event outcomes. Although it has been established that 10 - 25 events are required for each covariate placed in a survival regression model, this imprecision my result in too many patients being recruited, excessive tissue being examined and heightened costs.

2.7.1.5 Limitations of post-hoc power calculations

Failure to reject the null hypothesis will always raise the question of adequacy of power and sample size of the study. Although post-hoc power calculations are available, they underestimate the biological effect size of the study and overestimate the sample size required (Hoenig, 2001). Whenever a test statistic is non-significant, the post-hoc power calculation will always be low. Similarly, a significant test statistic will always be associated with high power. Post-hoc sample size calculations using the effect size observed following the completion of a non-significant study will always suggest a larger

sample size even if failure to reject the null hypothesis is appropriate. Therefore, sample size calculations should be performed *a priori* or if required performed retrospectively using an *a priori* approach.

2.7.2 Data types and derivation

The principal objective of data presentation is demonstrating the features of a study to the reader. Data may be described as either quantitative or qualitative.

2.7.2.1 Qualitative data

Nominal data

Nominal data consists of unordered observations with no statistical relevance between the nominated groups. Examples include alive or dead, male or female or geographical areas England, Scotland or Wales. Binary outcome measures such as gender are also data categories in their own right and may be considered independent of the nominal data type.

Ordinal data

Ordinal data consists of more than two categories whereby there is a progressively ordered relationship. Examples include T stage 1, 2, 3, 4 or classification of age <60 / 61-69 / >70. The values of the groupings may be numerical but they have no quantitative significance and are thus considered as nouns.

2.7.2.2 Quantitative data

Numerically discrete

Numerically discrete data simply consists of counts between two ranges and are integers. An example includes the number of people in a study who have died or those that received a specific treatment.

Numerically continuous

Numerically continuous data are measurements that in theory could take any measurement within a range and may also consist of a fractional element. Examples include measurements of weight in kilograms or calculations of body mass index. There are however, continuous variables that can be represented as discrete variables such as age, which may be described as full years (66 years old) or fractional (66.25 years old). An

important distinction in continuous data types are interval and ratio variables. Ratio variables have a true zero, whereas interval variables have an arbitrary zero. Degrees Celsius is an interval scale. Degrees Kelvin is a ratio scale.

2.7.2.3 Derived variables

For ease of presentation or incorporation into inferential statistical tests, data may be derived from those originally captured. There are several ways of deriving data based on categorizing recorded variables, threshold values, reference curves of standard population data and transformed variables. All of these derivative methods have their own strengths and limitations. The two methods utilized in this thesis are categorisation and threshold derivation.

Calculated or categorical data

Calculated or categorical derivatives represent grouped continuous or closely ordered discrete data. Examples include age, which can be categorised into decades, or otherwise grouped arbitrarily such as <50 / 50-65 / >65. Height which can be dichotomized as above and below the median, or represented as equally sized groups such as quintiles.

Variables based on thresholds

These derived variables are based on accepted thresholds of the measured variable. Examples include age, which can be dichotomised as an adult (≥18 years old) or pensioner (≥60 years old for a woman and ≥65 years old for a man). Another method of threshold derivation is the dichotomisation of CRP as normal (≤ 10 mg/l) or high (> 10 mg/l), based on measurements in defined populations.

Assumption of derived variables

Derivative methods assume that data contained within each group is sufficiently similar to allow categorisation together, yet sufficiently different to its immediate ordinal neighbours to make the properties of each group different.

2.7.3 Data associations: dependence vs independence

An *association* is an inferred relationship between two sets of data. The basis of such a relationship and how it is inferred depends on the method of statistical analysis of the sample of data. Inferential statistical association tests aim to disprove, within an accepted

type I error rate, either an ordinal association across a range of data quantities (dependence) or a difference between the two data samples (independence). A statistical test for independence between two data samples is a different concept from statistical tests for independent samples which occurs when an interval dependent variable of two independent groups are compared.

Statistical tests for data dependency demonstrate a relationship between two data samples. These relationships may be classified graphically as linear or non-linear and inferred using tests for correlation and regression.

Statistical tests for independence look for significant differences between the data samples. These differences can be based on the means of each sample (Student t-test), the medians (sign test, one way ANOVA, Kruskal-Wallis test, Wilcoxon signed ranks test and the Wilcoxon rank sum test) or the observed distribution of categorical variables against their expected distribution (Chi-squared test, Fisher's exact test or the McNemar test for paired samples).

All the statistical tests identified above are associated with data assumptions that must be met if the test is to produce a reliable result. There is no single test available that covers all data types and statistical inferences required for hypothesis testing. The rationale, assumptions and limitations for commonly used association tests are discussed below.

2.7.3.1 Statistical testing for data dependence

Continuous data comparisons

Graphical representation of data relationships

When looking for relationships between two sets of continuous data, a scatterplot is useful to determine the appropriateness for implementing an inferential statistical test. Data sets are labeled as independent (hypothesised causative factor) or dependent (the response factor) and are placed on the horizontal and vertical axis respectively.

Based on the scatterplot, data relationships may be described as no relationship, linear, quadratic, monotonic or exponential to name just a few. The decision on which inferential statistical method to be employed for assessing data relationships is based on the type of relationship observed on the scatterplot. The most common methods of assessing data dependency is through correlation or regression.

Correlation

One method is to look at how the data samples relate to each other across their distributions. If there is a dependent relationship between the ordinal increase in the measured data samples, then a correlation is said to be present. Correlation aims to measure the degree of association between two numerical variables. The two numerical data are described as the outcome variable and the exposure variable. The resultant correlation coefficient ranges from -1 to +1 with 0 demonstrating no relationship, -1 a perfect inverse linear association and +1 a perfect positive linear association. There are different methods of determining the correlation coefficients depending on the distribution of the data. It should alays be remembered that significant correlation does not of itself imply or even support causation in any particular direction, or at all.

Pearson correlation coefficient

Pearson correlation coefficient (PCC) is based on the covariance of the two variables divided by the product of their standard deviations (Pearson, 1896). The test may be applied to a population or a representative sample of the studied population. It is a parametric correlation test as it assumes normal distribution of both the outcome and exposure variable. The basis of this test is to address the hypothesis that there is a linear relationship between the two variables.

Assumptions

1. The variables must be continuous data measurements
2. The variables need to be normally distributed
3. The variables need to be of similar variance (homoscedasticity)
4. There needs to be a minimum of outliers in the variable data sample
5. There needs to be a linear relationship between the variables

Limitations

In clinical practice, it is not always possible to meet the assumptions of Pearson’s correlation coefficient test. Biological measurements such as weight or expression of a protein may not be normally distributed. Furthermore, removing outliers may not be appropriate as there may to be reasons for such observations which should not be observed.

Spearman's rank correlation coefficient

The Spearman's rank correlation coefficient (SRCC) is a nonparametric measure of dependence between two data samples (Spearman, 1904). It measures how close the association between the two data samples follows the monotonic function. The SRCC is based on ranked variables rather than the raw ordinal measurements of the two data samples.

Assumptions

1. Variables must be ordinal, interval or a ratio
2. There is a monotonic (steadily increasing or steadily decreasing) relationship between the two data samples

Limitations

The SRCC pays no regard to the magnitude of the observations and simply places the values in rank order. In smaller sample sizes, outliers may significantly change the ranking of the measurements and thus the correlation measurement observed. Despite the SRCC test offering a less sophisticated measure of correlation, it does not require numerous assumptions and therefore it is suitable for a broader range of biological investigations.

Regression

Statistical regression analysis is a process of estimating a relationship between dependent and independent variables. It aims to quantify how the value of a dependent variable changes when the independent variables are varied. The estimation or dependent variable is a function of the independent variable, which is often called the regression function. Multiple regression techniques that can be used for relationship forecasting, causal effect relationship determination and time series modeling.

Linear regression

Linear regression predicts the value of a dependent variable from an independent variable when both are linearly related (Bland, 1994a, Bland 1994b). The linear regression model takes the form $\boldsymbol{y = \alpha + \beta x + \varepsilon}$ where $\boldsymbol{\alpha}$ is the y intercept, $\boldsymbol{\beta}$ is the slope of the regression and $\boldsymbol{\varepsilon}$ is the error term. Certain assumptions of the data must be met.

Assumptions

1. A linear relationship between the dependent and independent variables

2. Homoscedasticity of the errors
3. Statistical independence of the errors
4. Normality of the error distribution
5. Lack of multicollinearity of the predictors
6. Minimal outliers

Limitations

Linear regression is most powerful when performed on continuous data and is less useful with ordinal or categorical data. If the data are not linearly related, then a different test is required. In addition, outliers have a large effect on the model and their inclusion must be limited as they may generate unreliable results.

Logistic regression

Logistic regression is a form of regression analysis where the dependent variable is categorical. Logistical regression is binary when the dependent variable is binary or multinominal when the dependent variable has more than two categories. Logistic regression models the relationship between a categorical dependent variable and one or more independent variables by estimating the probability using a logistic function.

Assumptions

1. Dependent variable is binary or ordinal
2. Only meaningful variables should be included
3. Independence of observations
4. Adequate sample size
5. Linearity of the independent variables as well as log odds

Limitations

Logistic regression requires larger samples sizes for reliable results. One rule of thumb for sample size estimation is to include approximately 30 cases for each predictor variable studied. The study also needs sufficient numbers in both or all categories of the dependent variable.

Multiple/Stepwise regression

Multiple regression analysis is performed when there are three or more measured variables in a regression analysis model. Multiple regression analysis may be performed for

prediction of independence of associations. Data variables are introduced into the model in a stepwise manner. Forward selection involved starting with no variables and introducing and comparing covariates until no further improvement in the predictive power of the model is made. Backward elimination involves including all candidate variables in the model and removing the covariate that is least significant until the model includes only independent variables. This method of examining relationships is associated with a reduced type I error rate and therefore no correction of the alpha value is required. Despite these advantages, many data assumptions that must be met to ensure reliable results.

Assumptions

1. Minimal number of outliers
2. Adequate sample size
3. Linear relationship
4. Multivariable normality
5. No auto-correlation
6. Homoscedasticity
7. Minimal multicollinearity

Limitations

The main limitation of this statistical model is its unreliability in small sample sizes. Although it offers information on relationships, it does not offer any information on causality and this is a general limitation of all relationship tests.

2.7.3.2 Statistical tests for data independence

Continuous data comparisons

The aim of determining statistical independence is to reject the null hypothesis that the two samples are statistically similar (ie derive from the same population). In terms of continuous data types, the aim is to compare the two data samples across their range of ordinal magnitudes. The inferential tests that are employed to assess this are dependent on the distribution of the data samples. Data samples that are normally distributed or can be normalised by a data transformation can be examined using parametric tests of the mean and those that do not approximately conform to data normality can be examined by non-parametric tests of the median. Each test has certain assumptions and limitations and these are discussed below.

Differences in the mean

One-sample t-test

The one-sample t-test is performed when we want to know if our data sample comes from a particular population. The one-sample t-test compares the mean of the sample with the known population mean whilst allowing for the expected sampling error. This test may be useful when assessing if a test cohort is representative of the disease cohort seen in routine clinical practice.

Assumptions

1. Continuous data type
2. The study population is normally distributed
3. The data sample represents random sampling from a defined population

Limitations

The main limitation of the one-sample t-test is that the population mean of the studied variable may not be known, for instance as a consequence of geographical variation, not yet fully characterised in the desired location of study. This may result in inappropriate rejection of the null hypothesis, when in reality the patient sample is representative of the population of interest. In addition, the one-sample t-test does not allow for inferential statistical analysis of two data samples.

Paired t-test

The paired t-test compares the means of two populations where observations in one sample can be paired with those in another sample. This test may be used to assess if a significant difference exists between before and after observations or two different methods of measurement on the same subject. The t statistic is the mean of the difference divided by the standard error of the mean difference. This follows a t-distribution with n-1 degrees of freedom. For this test to offer reliable results, certain assumptions must be met.

Assumptions

1. Continuous data type
2. The difference between the paired samples is normally distributed
3. The pairing of data occurs prospectively not post-hoc
4. The paired data samples must be independent

Limitations

When the differences between the two measurements are not approximately normally distributed, the inferential statistic is not entirely reliable and another method of assessment is required. The basis of the paired t-test is that the paired measurements are independent of each other but relate to the same subject. There are occasions when an assessment of difference in measurements between two populations is required when all subjects are exclusive and therefore a different statistical test is required.

Unpaired t-test

To compare the means between two populations, the unpaired t-test may be used. The test is based on the t-statistic, which is given by the difference between the means of the two samples divided by the standard error of the difference of the means of the two samples. The statistic follows a t-distribution with the sum of the observations in the two samples minus 2 degrees of freedom.

Assumptions

1. Continuous data type
2. The data samples are normally distributed
3. The data samples must be independent

Limitations

The main limitation of the unpaired t-test is similar to other t-tests in that data needs to be normally distributed (or can be normalised by a suitable transformation). In addition, the unpaired t-test is limited to the analysis of means between two groups and therefore a different test is required if more that two groups are being studied.

One-way analysis of variance (ANOVA)

One-way ANOVA is used to determine if a significant difference in the mean of three or more independent and unrelated groups exists (Fisher, 1918). The benefit of performing an ANOVA F-test over multiple t-tests is that the probability of obtaining a type I error is equal to α regardless of the number of groups being studied.

Assumptions

1. Dependent variable is a continuous data type
2. Independent variable consists of two categorical and independent groups

3. Significant data samples should be removed
4. Data samples must be independent
5. The dependent variable should be approximately normally distributed for every independent categorical variable
6. There must be equal variance between the proposed data populations

Limitations

One-way ANOVA does not identify which groups were significantly different and is thus called an omnibus test statistic. Although it is relatively robust against the assumptions of data normality, there is a risk of unreliable results and an alternative non-parametric test is required.

General limitations of statistical tests comparing means

When data is not normally distributed, the mean may disproportionately lie to the right of left of the data midpoint and is thus unreliable when entered into inferential statistical tests of the mean. Data transformation can be performed when violations of the normality assumptions exist, however, different statistical tests may be required. The t-tests are based on a comparison of the mean between two groups, however, there may be occasions when more than two groups are studied and an alternative statistical test is required.

Differences in the median

Sign test

The sign test is a non-parametric test that examines the direction (+ or -) of the observation against the median. The maximum of r (of the +ve or –ve observations) follows a binomial distribution for p=0.5 and $n=n'$. It can be used when a data sample is not normally distributed, transformation of data is not normally distributed or the sample size is too small to ascertain the distribution.

Assumptions

1. The differences between the paired data samples are independent
2. The difference measurement comes from the same population of samples
3. The values for the paired measurements are ordered chronologically so that differences observed are true meaningful observations

Limitations

The sign test makes few assumptions of the distribution of the data but it may lack statistical power when compared to alternative tests.

Wilcoxon signed ranks test

The Wilcoxon signed ranks test is a non-parametric test of the null hypothesis that the median of a distribution is equal to a specified value (Wilcoxon, 1945). It can be used on a single data sample as well as paired sample sets. In the paired test, the differences are ranked and labeled with their respective sign (as in the sign test). The minimum value for W (W-, W+) is chosen and using tables of critical values for the Wilcoxon signed rank sum a probability for observing this value of W is obtained. The single sample test is similar to the paired sample whereby the observed data is compared to a hypothesized median M.

Assumptions

1. Paired data are sampled from the same population
2. The data are ordinal
3. Paired data samples are chosen randomly and independently

Limitations

Data values of the same ordinal magnitude occupy the same rank thus diluting the inferential ability of the test. The sign test and the Wilcoxon signed ranks test rely on the data sample being pared to a hypothesized median or paired independent samples. On occasions, inferential tests of the median of two independent data samples are required and therefore a different statistical test is required.

Wilcoxon rank sum test / Mann-Whitney U test

The Wilcoxon rank sum test or Mann-Whitney U test is a non-parametric inferential statistical test for the comparison of medians from two independent data samples (Mann, 1947). The test is based on the ranking of all observations in order of ordinal magnitude, with identical values given average rankings. The test is mathematically identical to conducting an unpaired t-test except with ranked values.

Assumptions

1. The dependent variable is a continuous or ordered data type
2. The independent variable is comprised of two independent categorical groups

3. The observations contained within the data sample are independent

Limitations

Similar to all non-parametric inferential statistical tests, the Mann-Whitney U test lacks power when compared to its parametric alternatives. This is due to the methodology of analyzing ranks rather than the ordinal magnitude of the observations.

Kruskal Wallis test

The Kruskal Wallis H test is a rank based non-parametric test to determine if there is a difference between three or more groups (Kruskal, 1952). It may be viewed as an extension of the Mann-Whitney U or the non-parametric version of the one-way ANOVA.

Assumptions

1. Dependent variable is a continuous data type
2. Independent variable consists of two categorical and independent groups
3. Data samples must be independent

Limitations

Similar to the one-way ANOVA, the test is an omnibus test and cannot identify which group is significantly different from the others. As with all non-parametric inferential tests, the use of ranks may reduce the statistical power of the test.

General limitations of statistical tests comparing medians

The inferential statistical tests of the medians of different groups have the benefit of not assuming normality of the test data. They have the added benefit of being more reliable when normality of data is uncertain. Given that they are based on the ranks of the data rather than their ordinal magnitude, they may offer weaker inferential statistical power than their parametric alternatives.

Categorical data comparisons

There are numerous tests available to analyse categorical data and detailing each one is not in the scope of this thesis. The most commonly used categorical statistical hypothesis tests are the chi-squared tests. A chi-square test is any hypothesis test where the sampling distribution of the test statistic follows the chi-squared distribution when the null hypothesis cannot be rejected. Test statistics that follow the chi-squared distribution are

assumed to arise from independent normally distributed data. Therefore, the chi-square test may be used in two hypothesis testing situations; goodness of fit tests and tests for independence. Goodness-of-fit tests estimate how closely observed distributions are related to expected distributions, and independence tests estimate if two random variables are independent. There are several versions of the chi-square test, however, the Pearson's chi-squared test is the most commonly used.

Pearson's chi squared test

Pearson's chi-squared test aims to establish if the difference in observed frequencies between two categorical data samples arose by chance (Pearson, 1900). The test for independence, commonly expressed in a contingency table, simply determines if a significant difference exists between the observations and does not offer any information about the direction or magnitude of the difference. The final significance value is an approximation of the test statistic to a theoretical chi-squared distribution with certain degrees of freedom.

Assumptions

1. The data sample is random and that the probability of selection is equal to that of the population from which the sample has been taken
2. The observations are independent
3. Adequate data sample to reduce the chance of type II reporting errors
4. Expected values of at least 5 in each cell

Limitations

The main limitation of Pearson's chi-squared test is that it is sensitive to the size of the sample and therefore a weak relationship may be observed as statistically significant. If small values are expected within cells, such as individual cell counts of 5 or less for 2x2 contingency tables or 20% of the fields with counts less than 5 in larger tables, the significance statistic is unreliable. This is due to the basis of the test being an approximation of a theoretical chi-squared distribution that becomes established as the sample size becomes infinite. In these situations, a different inferential test is required.

Fisher's exact test

Fisher's exact test calculates the significance for the deviation from the null hypothesis exactly, rather than relying on an approximation to the theoretical chi-squared distribution,

which is used in the chi-squared test (Fisher, 1922). The test is based on the observation that the probabilities of fixed marginal totals can be estimated using a hypergeometric distribution with four classes.

Assumptions

1. Categorical data type
2. Data observations are independent
3. The count values in the cells are fixed or conditioned

Limitations

The main limitation of the Fisher's exact test is that it simply reports if the distribution of observed events are independent of each other and does not offer any information about the direction or magnitude of the difference.

McNemar's test

McNemar's test is an inferential statistical test for significance of change on dichotomous dependent variables between two groups of paired nominal data (McNemar, 1947). The test is based on the chi-square statistic with the cells of interest representing the change in dichotomisation between the primary and secondary observation.

Assumptions

1. One categorical dependent variable with two categories along with one categorical independent variable with two related groups
2. The two dependent groups are mutually exclusive
3. The sample data represent a random sample from the studied population

Limitations

McNemar's test is not as statistically powerful as other parametric tests. In addition, when faced with smaller sample sizes (discordant pairs >10), the McNemar's test loses statistical power and an alternative test is required.

2.7.3.3 Limitations of data association tests

Association between two variables does not imply causation. The delineation of causation using associations alone is difficult given the complex nature of natural science. An

association between A and B can be explained by three potential scenarios, firstly A causes B, secondly B cause A and thirdly A and B are caused by an external factor C. It is possible to develop statistical models that will establish which of the co-factors are independently associated with a particular variable, however, this is limited by the inclusion or exclusion of important co-factors and the sample derived to develop the statistical model. Given the nature of experimental science, not all the co-factors in a complex multifactorial relationship can be studied and therefore apart from associations, causality cannot be determined without doubt.

2.7.4 Adjustment of multiple comparisons

Multiple comparisons arise when a number of inferential statistical test are performed on paired groups or a single dataset. Using a p-value of 0.05, the chance of incorrectly rejecting a true null hypothesis is 5% (type I error). Performing 100 comparison tests on the same dataset would on average result in a false rejection of the null hypothesis in 5 cases. Similarly, one hundred 95% confidence intervals will likely not cover the population parameter in 5 cases. To ensure that the chance of a type 1 error is sufficiently diminished, a multiple testing correction can be performed.

The Bonferroni correction

The Bonferroni correction method is used to counteract the problem of multiple comparison testing (Bland, 1995). The test is based on a researcher testing *n* hypotheses at a significance level of 1/*n* of α, thus:

$$Bonferroni\ correction = \alpha / n$$

A limitation of the Bonferroni correction is that the reduction in type I errors is associated with an increased chance of not rejecting the null hypothesis when in fact this is false (type II errors). Therefore, each comparison with a modified $\alpha < 0.05$ needs to be interpreted within the context of the study, its strengths, limitations and other significant results.

2.7.5 Univariable survival analysis

Survival analysis is a process of examining data where the outcome variable is the time observed between the start of the study and an event of interest. Within this thesis, the events of interest are disease recurrence, death or censorship, which occurs when the information about a patient's survival is incomplete. Censoring is encountered during two

instances; firstly when patients have been lost to follow up and secondly, when patients have not undergone a defined event at the point of study completion (Altman, 1998).

The inferential quality of survival analysis is dependent upon accurate ascertainment of the outcome event and appropriate selection of statistical methodology. The data required for survival analysis is the time between entry to the study and event occurrence or censorship as well as the defined event status. Different survival methodologies are associated with inherent mathematical assumptions and thus, inaccuracies in the data or inappropriate choice of testing methodology for the type of data may lead to erroneous results.

There are several methods for analysing the survival outcomes for patients in observational cohort studies. No single test offers a comprehensive inferential analysis of outcome and combinations of tests are often used. Each test has its own pre-defined assumptions and limitations which are discussed below.

2.7.5.1 Follow-up, recurrence, death and study endpoints

Two primary end points that are of particular clinical interest in treating patients with cancer; time to disease recurrence and overall survival. There are two main challenges, predicting who will develop recurrence or who will die of their disease and the time taken to reach these endpoints.

In practice, precisely defining these endpoints may be difficult. Date of death is a precise endpoint, but determining what constitutes a cancer-related death is not always clear. The cause of death given on a death certificate may be inaccurate and all-cause mortality has been widely used to avoid this difficulty. Similarly, determining the time of disease recurrence is difficult as recurrence may be ascertained at different times due to surveillance (asymptomatic) or as emergencies (symptomatic). Close follow-up regimens, which might include regular surveillance CT scans, reduce the chances of symptomatic presentation as patients are identified earlier. Limitations in defining the date of disease recurrence are unavoidable and results have to be interpreted with caution.

2.7.5.2 Survival probabilities (Kaplan-Meier methodology)

The Kaplan-Meier method is a non-parametric statistical method for estimating the survival function of 2 or more groups (Kaplan, 1958). The test is constructed by defining a survival event such as death or recurrence and patients survive until the occurrence of this event, or they are censored. The proportions of patients surviving at each time point is

calculated over the survival period. The Kaplan-Meier method aims to estimate the probability of survival for a member of the population from which the sample group is drawn. The results of this test can be displayed as a Kaplan-Meier survival plot or life tables. To achieve reliable information from the Kaplan-Meier survival method, certain assumptions have to be met.

Assumption

1. Patients who are censored have similar survival prospects to those that remain in follow-up
2. The survival probabilities are the same for those recruited at any time point
3. The event occurred at the specified time

Limitations

Although the Kaplan-Meier graph offers useful information on the probabilities of survival events occurring at a particular time point, it does not offer any significance information for hypothesis testing and thus additional tests are required such as the logrank test (Bland, 1998).

2.7.5.3 Logrank test

The logrank test is a hypothesis test, which compares survival distributions between studied groups (Mantel, 1966). It tests the null hypothesis that there is no difference between the groups in the probability of death at any point in time. The basis of the test is comparing the observed number of deaths to the predicted number of deaths on the basis that there is no difference between the groups. At the point of the first event, the risk of death is 1/n, where n is the total number of participants in the study. The sum of patients in each group is then multiplied by the risk of death for the entire cohort. The same calculations are performed each time an event occurs. When a patient is censored, that patient is at risk of dying up to the point of censoring but not afterwards. Using the chi-squared test, the expected and observed deaths in the two groups are analysed and a p-value calculated (Bland, 2004). In order to perform a logrank test several assumptions need to be met.

Assumptions

1. Sample is chosen randomly

2. Definition of survival is consistent between all subjects
3. Baseline and survival rate are do not change over time
4. The survival time of the censored subjects are the same as the remaining subjects
5. The variable designating group membership is independent of other patient covariates
6. There is no correlation between covariates

Limitations of the logrank test

The logrank test is merely a measure of significance and does not offer any information on the magnitude of difference between the groups. Furthermore, it does not offer any information on the confidence interval survival association. A further limitation is that statistical significance will only be detected if the risk of the event occurring is consistently higher in one group compared to the other. Therefore, survival data where the Kaplan-Meier curves cross will be unlikely to lead to a significant result and additional statistical testing is required.

2.7.6 Multivariable survival analysis

Whilst the logrank test identifies statistically significant differences between groups, without information on the magnitude of the difference, the clinical importance of the biomarker on survival is not clear. The previously discussed survival methods are also limited by their failure to control for confounding effects of covariates on the observed survival function. In clinical practice, it is common for several known or unknown confounding factors to influence survival. Therefore, whenever investigating the relationship between a particular biomarker expression and survival, it is desirable to adjust the survival association for other confounding factors. Several methods have been proposed for modeling survival data and are based on the distribution of the survival times (Bradburn, 2003).

2.7.6.1 Cox proportional hazards model (Semi-parametric)

The Cox proportional hazards model estimates a studied variables' effect on survival after adjustment for other confounding factors (Cox, 1972). It therefore simultaneously explores the effects of several variables on survival. In addition, it can also offer univariable survival information through the calculation of a hazard function. The Cox proportional hazard model is a semi-parametric test that offers two main features; a hazard function and the multiple regression model.

The hazard function is the probability that a participant will experience an event within a specified time interval, given that the individual has survived up to the commencement of that time period. The hazard function is estimated by dividing the number of individuals experiencing the event in that particular time interval by the number of participants surviving at that time multiplied by the interval width (Cox, 1972).

The Cox regression model differs from ordinary regression as the covariates are used to predict the hazard function. It considers the risk sets of subjects still being followed up at each time a survival event occurs. At each event point, the values of the covariates for the participant undergoing the event is compared to those for all the surviving participants remaining in follow up.

Assumptions

1. That mechanisms giving rise to censoring are independent of the probability of an event occurring
2. The model relies on the relationship between the dependent and explanatory variable being proportional over time
3. The studied variable and other covariates contribute linearly to the natural log of the hazard ratio

Limitations

The Cox proportional hazards model assumes that the relationship between the studied covariates and the hazard function remains constant during follow-up. Many clinicopathological factors such as tumour stage remain unchanged, however, changes to modifiable factors, such as body mass index, may inadvertently violate the assumptions of the model and may lead to incorrect conclusions. The reliability of the Cox model is based on strict adherence to the underlying assumptions and potential covariates should undergo checking to confirm compliance prior to model construction, which takes the form of various tabular and graphical outputs, which require a reasonable grounding in statistical methodology.

2.7.6.2 Parametric proportional hazards model

Parametric proportional hazard models resemble the Cox proportional hazard model but the hazard is taken to follow a specific distribution. Such models are named after the distribution of the hazard function including exponential, Weibull and Gompertz

proportional hazard models. Although these models offer improved survival prediction when compared with semi-parametric Cox models, their application is are limited by their parametric nature and they are not appropriate for all datasets.

2.7.6.3 Accelerated failure time models

The accelerated failure time model (AFT model) is a parametric survival model that assumes the effect of a covariate is to accelerate or decelerate the survival course of a disease by a constant relative amount (Bradburn, 2003). AFT models are fully parametric and can be performed on multiple distributions for the log of $T_{0,}$ (the unmoderated distribution of T(time)). The most common distributions are log-logistic and Weibull, but normal, gamma and inverse Gaussian may also be used (Bradburn, 2003). In contrast to proportional hazard ratios, AFT models interpret the effect size as a time *ratio*.

Assumptions

1. The mechanism resulting in patient censoring is independent of the probability of the event occurring
2. Survival times follow a predefined distribution
3. The model relies on the relationship between the dependent and explanatory variable being proportional over time
4. The effect of the covariate on survival is fixed and does not change over time

Limitations

Although the parametric nature of this modeling method offers superiors prediction, it is limited to assessing data with a specified survival time distribution. Non-parametric modifications of the AFT model have been proposed, but not widely accepted.

2.7.7 Biomarker quantification: Assessing reproducibility of measurements, including subjective assessments

To reduce reporting bias, data measurements require independent validation. Validity of analysis depends on accuracy and precision of the test data, which should be reproducible when assessed by an independent observer or another assay. Depending on the data type of the measurement, it is unlikely that exact agreement will be observed between two raters. Whilst allowing for limitations in scientific techniques, quantification of inter-observer

agreement offers some information about relative validity of data. Methods of quantifying inter-observer agreement for different data types are discussed below.

2.7.7.1 Cohen's kappa coefficient

Cohen's kappa coefficient measures the inter-observer agreement for categorical data (Cohen, 1960). The κ statistic is calculated by dividing the difference between the observed agreement and the agreement that would be expected by chance alone by 1 minus the hypothetical chance agreement. When there is complete agreement $\kappa = 1$, however $\kappa = 0$ if there is no agreement other than that which could occur by chance. $\kappa < 0$ implies a systematic disagreement.

Limitations

Cohen's kappa coefficient is based on agreements between raters in categorising data into certain groups; therefore, measurements that are of a continuous data type require a different test.

2.7.7.2 Interclass correlation coefficient – unordered pairs

The interclass correlation coefficient (ICC) assesses the consistency of measurements between observers or assays measuring the same quantity. The test is based on dividing the variance of the two measurements divided by the sum of the variance between the two measurements plus the measurement error variance. The ICC may range from 0 no agreement to 1 complete agreement. It requires a data type, which is at least interval if not ratio.

Limitations

This method of ICC assumes that the data are ordered pairs and therefore only applies to a set of two measurements. For assessment of reliability between more than two observers, a different statistical test is required.

2.7.8 Rationale for choice of descriptive and inferential statistical methodology

2.7.8.1 Statistical software

All data analysis was performed using SPSS (version 20.0 for Mac).

2.7.8.2 Sample size calculations

Rationale for determining effect size

The AJCC/UICC TNM staging system remains the standard prognostic marker for predicting 5 year survival following surgery for colorectal cancer. Outcomes vary by stage with stage I having a 95% 5 year survival compared to 80% in stage 2, 63% in stage III and 7% for stage IV. 5-year survival for all patients undergoing potentially curative resection is 60%. New candidate biomarkers should at least offer similar effect sizes to TNM staging system and therefore for the purpose of sample size calculations, a 15% difference from a baseline of 60% 5 year overall and recurrence free survival was sought. In addition to survival, 15% difference was sought for comparing proportions across categorical data.

Rationale for determining type 1 and type 2 error rates

The currently accepted rates for type 1 and type 2 errors are $\boldsymbol{\alpha}$ = 0.05 and $\boldsymbol{\beta}$ = 0.2 respectively. The adjustment of $\boldsymbol{\alpha}$ for multiple comparisons is given through out the text and varies according to number of tests performed.

Sample size calculation – effect size

Using a study design of two independent groups, a dichotomous primary endpoint with an $\boldsymbol{\alpha}$ = 0.05 and $\boldsymbol{\beta}$ = 0.2 and an anticipated effect size of 15% from a baseline of 60% the sample size required is $\boldsymbol{n}$ = 346. Adjusting $\boldsymbol{\alpha}$ = 0.0025 for twenty comparisons results in a sample size requirement of $\boldsymbol{n}$ = 658.

Patients included in the studies reported in this thesis were not screened for clinicopathological factor determination prior to analysis. This approach aims to reduce bias and improve reliability of the results. To ensure adequate numbers of patients for subgroup analysis, the overall volume of patients in the study will need to be significantly larger that n=677. Calculations for this will take the form of dividing $\boldsymbol{n}$ = 658 by the proportion of patients in the subgroup.

Sample size calculation – number of events

As previously discussed, approximately 10 – 25 events are required for every covariate in a survival model. Based on the assumption that survival models will include a maximum of 12, which equates to 120 – 300 required events. Given the 5-year survival rates discussed above, a sample size of $\boldsymbol{n}$ = 658 would include 394 events. Examining survival outcomes in subgroups is also likely to require a greater number of patients, however, depending on

the anticipated rate of events in each subgroup this figure could be substantially smaller or larger.

2.7.8.3 Continuous data comparisons

Data dependence

All continuous data types were graphically represented using a Q-Q (quantile-quantile) plot to test for data normality. To determine if a correlation between continuous data types exist, Pearson's correlation coefficient was employed. For data that are not normally distributed, Spearman's rank correlation coefficient was used.

Data independence

To assess independence between the distributions of continuous data stratified by a categorical biomarker unpaired t-tests were used. For data that is not normally distributed, the Mann Whitney U test was employed. Whenever possible, parametric tests were used for their superior statistical power.

2.7.8.4 Categorical data comparisons

For all categorical data types, the Pearson's chi-squared test was used to assess for independence between the proportions observed between the two variables. Given the number of patients contained with the cohorts, Fisher's exact test is not required.

2.7.8.5 Survival data and follow up protocol

Follow-up

Follow-up was adapted to the treatment being received by individual patients. Patients who did not undergo adjuvant therapy and who were placed into the surveillance program underwent the following follow up:

- Clinical review at 1, 3, 6, 12, 24, 36, 48 and 60 months following surgery
- CT thorax, abdomen and pelvis at 12, 24, 36, 48 and 60 months
- Colonoscopy at 12 and 60 months

Patients who underwent adjuvant therapy had additional clinic and radiological follow-up. Patients who developed symptoms, which on clinical review were concerning for disease recurrence had additional radiological and or endoscopic investigations.

Choice of study endpoints

Given the important limitations associated with disease-specific survival, overall survival has been chosen as the primary survival endpoint in these studies. The biomarkers studied are specific to the tumour and may not affect overall survival, which is affected by both tumour and non-tumour related disease processes. Overall survival may underestimate the effect of the biomarker on survival and therefore time to recurrence has been retained as a secondary survival endpoint. The methodologies for determining survival endpoints are detailed below.

Date of recurrence and death

On review of clinic letters, CT scan reports and endoscopy results, the date of recurrence was defined as the date that one or other diagnostic modality identified disease recurrence. Using a combination of case notes review and death certificate reports, the date of death was determined by the date that death was certified. Patients who were right censored had a date of recurrence and date of death that represented the date they left the study. For those that died but did not have proven recurrence, their date of death was their censoring point. Thus the endpoint for recurrence was binary: recurrence present no=0, yes=1 and the endpoint for survival was also binary, dead no=0, yes=1. The calculation for measurements of time to recurrence and survival are shown below.

For those with recurrence:

Time to recurrence (months) = date of surgery – date of recurrence endpoint (date of recurrence or date of death or date of censorship)

For those without recurrence:

Time to recurrence (months) = date of surgery – date of censorship (date of death or 60 months follow up)

For those that died:

Overall survival (months) = date of surgery – date of death

For those still alive:

Overall survival (months) = date of surgery – date of censorship

2.7.8.6 Survival association tests

Descriptive analysis

Kaplan-Meier graphs were produced to demonstrate the relationship between studied biomarkers and the survival endpoints chosen. Survival tables were included to demonstrate the number of patients present at each follow up stage. This combined approach offers a visual and tabular representation of proportionality of survival at each significant stage across the follow up range.

Univariable analysis

Cox proportional hazard along with the logrank test will be used to assess the difference in survival between the categories of the studied biomarker. The logrank test does not offer any information on the magnitude of difference between the two categories and therefore the Cox proportional hazard model was also utilized.

Multivariable analysis

A multivariable Cox regression model will be developed incorporating factors with a significance level of <0.1 and which are appear clinically relevant when considering data associations. Factors not naturally dichotomous were included in their continuous form to reduce the risk of imprecise results that can be seen when factors such as age are transformed into categorical data. All covariates will be entered into the model and eliminated in backwards-stepwise manner. Covariates demonstrating time dependency will be included in the model along with interaction terms between the covariates and (log) time as described by Bradburn et al (Bradburn, 2004).

2.7.8.7 Methods for assessing data assumptions

Data distribution

For the assessment of data normality histograms and Q-Q plots were graphed to allow visual inspection of the data. The Shapiro-Wilk statistic was also be used to support the observations seen in the graphed data. For continuous data comparisons, scatterplots were used to visually inspect the data linearity and to ensure that assessment with inferential correlation tests are appropriate.

Survival data

Assessing compliance with Cox proportional hazards model assumptions is different for continuous and categorical data. Not all statistical tests that assess data assumptions are available in SPSS Continuous variables were categorised into groups as supported by Bradburn et al (Bradburn, 2004b) and assessed using the following stepwise process.

Proportionality

1. Kaplan-Meier curves should gradually diverge; lines that cross were considered to have violated the proportionality assumption. Variables that interact with time may also demonstrate variability in the rate of events occurring over particular points in the follow-up phase.
2. Log(-log(survival)) plot – If the hazards are proportional then the stratum specific log(-log(survival)) plots will exhibit a constant difference and appear parallel.
3. Time-dependent covariate test – A covariate that has a time-dependent hazard ratio will appear as a significant result when the variable as a function of time is included in a Cox regression analysis.

2.7.8.8 Biomarker quantification and assessment of reproducibility

Weighted histoscore

The semi quantitative histoscore method has been described in section 2.6.6.1. This method of scoring has widely used by our team and many others. The histoscore ranges from a 0-300 and is calculated independently for nucleus, cytoplasm and membrane. Using a combination of Kaplan-Meier curves and Receiver-operator-characteristic (ROC) curves, thresholds for grouping biomarkers were chosen. The ICC was used to assesse the reproducibility of the biomarker quantification when 10% of the TMA cores were co-scored by an independent investigator blinded to primary scores and outcome measures. For the purpose of reliability, ICC's > 0.7 are classified as acceptable and > 0.8 are considered excellent.

Assessment of mismatch repair protein expression

Mismatch repair protein (MLH1, MSH2, PMS2 and MSH6) expression was established using UK NEQAS scoring guidelines. Expression is categorised as normal, patchy/weak or negative. Cohen's kappa coefficient was used to assess the inter-observer correlation for these categorical data.

3. The relationship between cancer associated inflammation, MSI status and survival

3.1 Introduction

Colorectal cancer is a heterogenous disease with variable patterns of genetic injury. Differences in the aberrant expression of particular genes have been associated with certain histological phenotypes. There are two primary genomic instability pathways: Microsatellite Instability (MSI) and Chromosomal Instability (CI) also referred to as Microsatellite Stability (MSS). Tumours arising by these pathways have a predilection for specific anatomical, histological and molecular biological features. In addition, they differ in terms of recurrence and 5 year survival rates. MSI tumours tend to be larger than their MSS counterparts but are more likely to be node negative, have a pronounced lymphocytic infiltrate at the tumours invasive edge and have a better prognosis (Soreide, 2006).

Cancer related inflammation has been described as a 7^{th} hallmark of cancer (Balkwill, 2001). The so-called hallmarks of cancer represent biological behaviors that have been identified as characteristic or fundamental to of the disease process. Tumours grow through a combination of dysregulation of cellular energetics, excessive cell proliferation, reduced apoptosis, loss of cell adhesion and invasion through tissue remodeling and dysregulation of angiogenesis (Balkwill, 2001). Inflammation plays an important part in all these factors. Despite the systemic inflammatory response (SIR) being associated with more aggressive malignant disease, infiltration by immune cells, particularly $CD8^+$ lymphocytes, at the advancing edge of the tumour has been associated with improved outcome (Naito, 1998). This suggests a complex relationship between tumour and host, which is both beneficial and detrimental to the patient. MSI tumours have been associated with a pronounced lymphocytic infiltrate within the cancer cell nests (Dolcetti, 1999), but it remains unknown if there is any association between MSI tumours and the presence of a SIR and what effect this has on patient survival.

The core hypothesis of this chapter is that the systemic inflammatory response will be significantly associated with MSI colorectal cancer. The previously reported poor survival associated with the SIR will not be observed in MSI colorectal cancer, due to the beneficial effect of the local inflammatory response. Finally, tumour MSI status, serum CRP and serum albumin will be significantly associated with survival outcome measures in stage II colorectal cancer.

3.2 Description of cohorts

3.2.1 Core clinicopathological factors

Core characteristics of the training and validation cohort are shown in table 3.1 along with the regional frequencies for the data variables studied during the same time period. Compared with the validation cohort and regional data, the training cohort had a higher proportion of male patients, elective presentations, rectal tumours and a shift towards more advances disease (Stage III 46.3% vs 35.8% and stage I 6.6% vs 15.7%). The validation cohort has similar frequencies to those in the regional data.

Table 3.1: Frequency of core clinicopathological factors between the training cohort, validation cohort and regional data (Nicholson, 2012)

Clinicopathological variables	Training cohort (Cohort 1) n=182	Validation cohort (Cohort 2) n=677	Regional data N=11,166
Age			
Median +/- IQR	70 (IQR 54 – 86)	72 (IQR 57 - 87)	72 (IQR 58-86)
Sex			
Female	42.3%	49.1%	49.9%
Male	57.7%	50.9%	50.1%
Mode of presentation			
Elective	95.6%	70.4%	83.3%
Emergency	4.4%	29.6%	26.7%
Tumour Site			
Colon	67.6%	78.2%	75.0%
Rectum	32.4%	21.8%	25.0%
Tumour site enhanced			
Right	37.9%	44.6%	
Left	29.7%	34.4%	Not available
Rectum	32.4%	21.0%	
Tumour Stage			
I	6.6%	15.7%	13.9% (18.2%*)
II	47.3%	48.6%	32.9% (43.0%*)
III	46.2%	35.8%	29.6% (38.8%*)
IV	0.0%	0.0%	23.7% (N/A*)

* Frequencies of TNM stages when only TNM stage I, II and III included.

3.2.2 Experimental clinicopathological factors

In addition to the basic patient demographics and tumour characteristics presented in section 3.2.1, tumour differentiation, serum CRP, serum albumin, tumour Klintrup score and MMR protein status were also evaluated. The statistical features of these biomarkers will be described in more detail later in this chapter; however, the distribution between categories of these clinicopathological factors are shown in table 3.2. The distribution of

tumour differentiation, Klintrup score and tumour MMR status is similar between the two cohorts.

All 182 patients in the training cohort had data available on serum CRP and albumin available. In the validation cohort, 533 patients had data on serum CRP expression and 563 patients had data on serum albumin expression. In the validation cohort, 26.6% of patients were hypoalbuminaemic compared with 13.7% of patients in the training cohort. In addition, 49.3% of patients in the validation cohort had a raised serum CRP compared to 45.1% in the training cohort.

Table 3.2: Comparison of proportions of experimental clinicopathological factors between the training cohort and validation cohort

Clinicopathological variables	Training cohort (Cohort 1) n=182	Validation cohort (Cohort 2) n=677	
Differentiation			
Well/Moderate	89.0%	90.3%	
Poor	11.0%	9.7%	
Serum CRP			
Normal	54.9%	50.7%	
High	45.1%	49.3%	n=533
Serum albumin			
Normal	86.3%	73.4%	
Low	13.7%	26.6%	n=563
Klintrup score			
High	31.5%	30.4%	
Low	68.5%	69.6%	
MMR status			
MMR-P	84.1%	84.2%	
MMR-D	15.9%	15.8%	

3.3 Training cohort

3.3.1 Systemic inflammatory response quantification

3.3.1.1 Serum CRP expression

Serum CRP was assessed in an accredited diagnostic biochemistry laboratory, using ELISA and reported in units of mg/l. Figure 3.1 demonstrates that the expression of serum CRP follows an exponential distribution (histogram, figure 3.1) with measurements ≥48 mg/l considered outliers (boxplot, supplementary figure 3.1, appendix 5.1). The measurements ranged from 5 – 200 mg/l with a median of 9 mg/l (IQR 5 - 25 mg/l)

(supplementary figure 3.1 appendix 5.1). Using the dichotomisation thresholds described by McMillan et al, 45.1% of patients were considered high expressers (>10 mg/l).

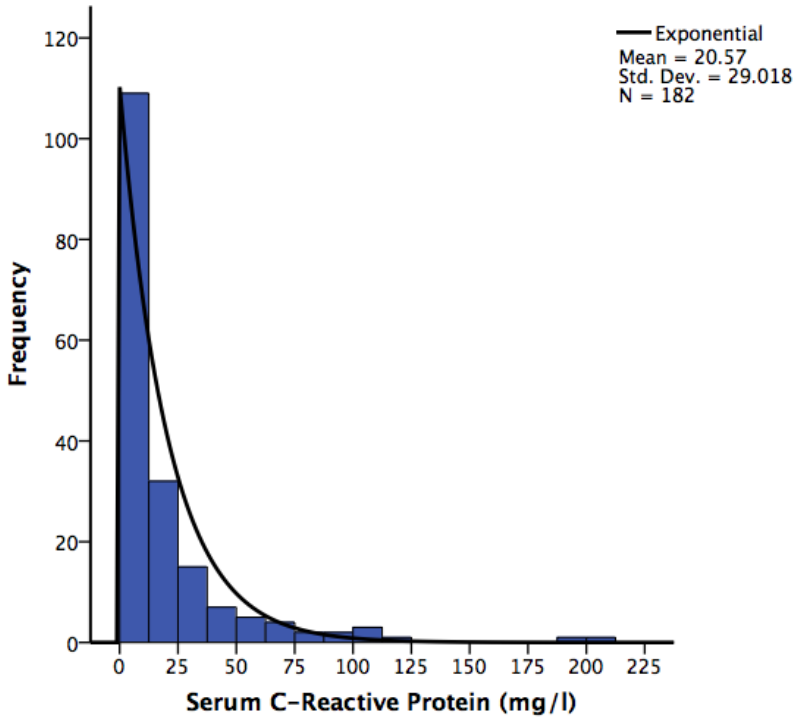


Figure 3.1*:* Distribution of measurements for serum CRP.

Histogram demonstrating the distribution of serum CRP measurements.

3.3.1.2 Serum albumin

Serum albumin was assessed in an accredited diagnostic biochemistry laboratory, using ELISA and is reported in g/l. Figure 3.2 demonstrates that the expression of serum albumin does not precisely follow a normal distribution (histogram and Q-Q plot, figure 3.2) which is supported by a Shapiro-Wilk statistic of 0.966, df 182, $p<0.001$. Measurements ranged from 23 – 52 g/l with median 39 g/l (IQR 33 - 45 g/l) (supplementary figure 3.2, appendix 5.1). Using the dichotomisation thresholds described by McMillan et al, 13.7% of patients were considered hypoalbuminaemic (<35 g/l).

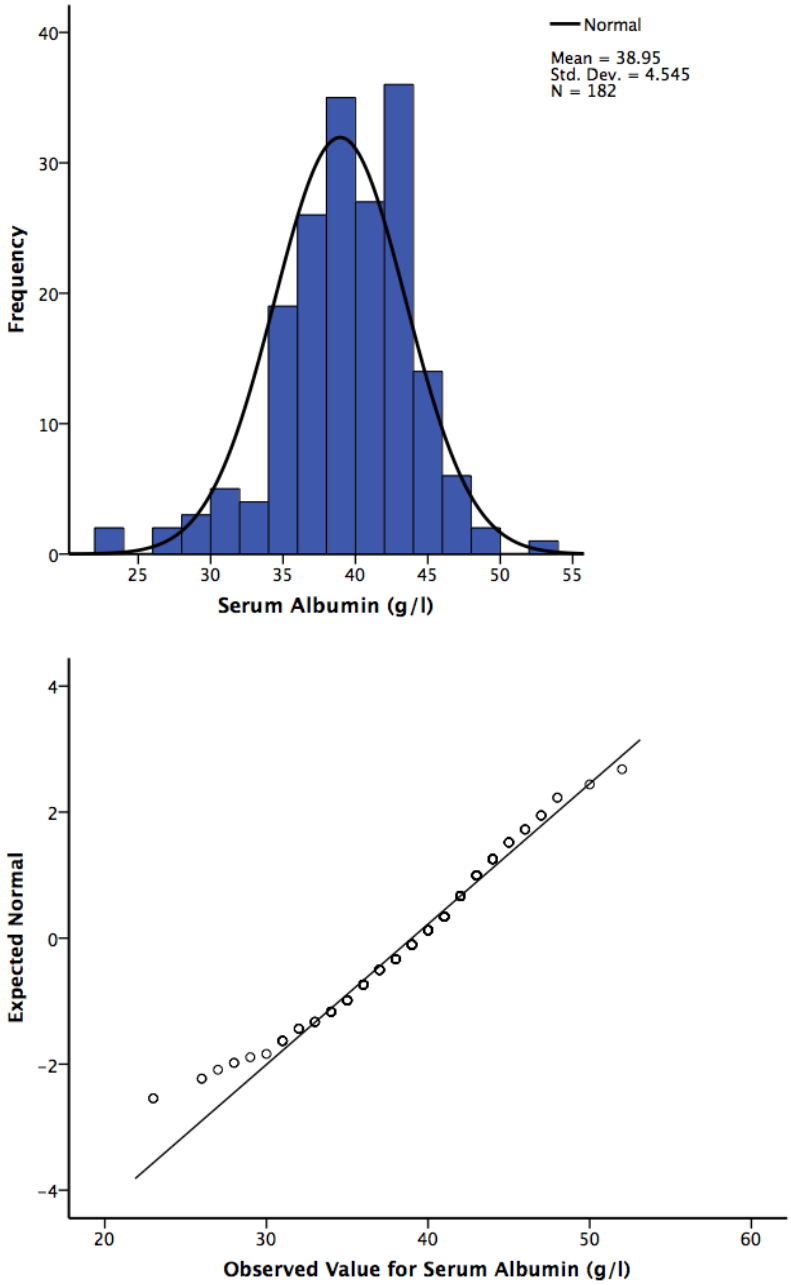


Figure 3.2: Distribution of measurements of serum albumin in patients with colorectal cancer

Histogram demonstrating the distribution of serum albumin measurements. Q-Q Plot of serum albumin measurements.

3.3.2 Mismatch repair (MMR) protein status determination

MMR protein expression status was determined by immunohistochemistry of mismatch repair proteins; MLH1, MSH2, PMS2 and MSH6. NEQUAS reporting guidelines were used when assessing expression. Tumours expressed MLH1 in 83% of cases, MSH2 in 96% of cases, PMS2 in 87% of cases and MSH6 in 94% of cases. Using NEQUS reporting

guidelines 15.9% were considered MMR deficient. Examples of MMR proficient (MMR-P) and deficient (MMR-D) tumours are shown in figure 3.3.

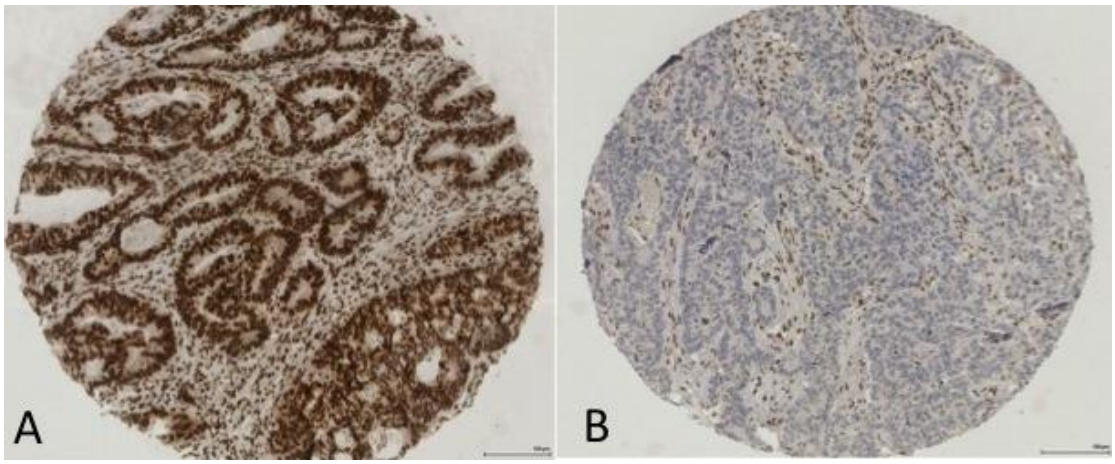


Figure 3.3: Immunohistochemistry for MLH1 protein in two different colorectal cancer

A.) TMA core showing a tumour with proficient MLH1 expression. B.) TMA core showing tumour with absent MLH1 expression. Note the normal expression in immune cells and fibroblasts in core B but no expression in the cancer cells.

3.3.3 The association of MMR protein status and clinicopathological factors

Patients with mismatch repair deficient tumours had lower levels of serum albumin, with a median of 37 g/l (IQR 31 – 43 g/l) compared with a median of 40 g/l (IQR 35 – 45 g/l) in patients with MMR proficient tumours (p=0.024, Mann-Whitney U, figure 3.4). This association, however, was not supported by chi-squared analysis when the variables were analysed as categorical data (p=0.076, Bonferroni adjustment p<0.003, table 3.3). On chi-squared analysis of categorical variables, right-sided tumour location (p<0.001) and poor differentiation (p<0.001) were significantly associated with mismatch repair deficient tumours following Bonferroni adjustment. When serum CRP was analysed as a continuous variable, there was no statistically significant difference between serum measurements when stratified by MMR protein expression (p=0.109, Mann-Whitney U, figure 3.4).

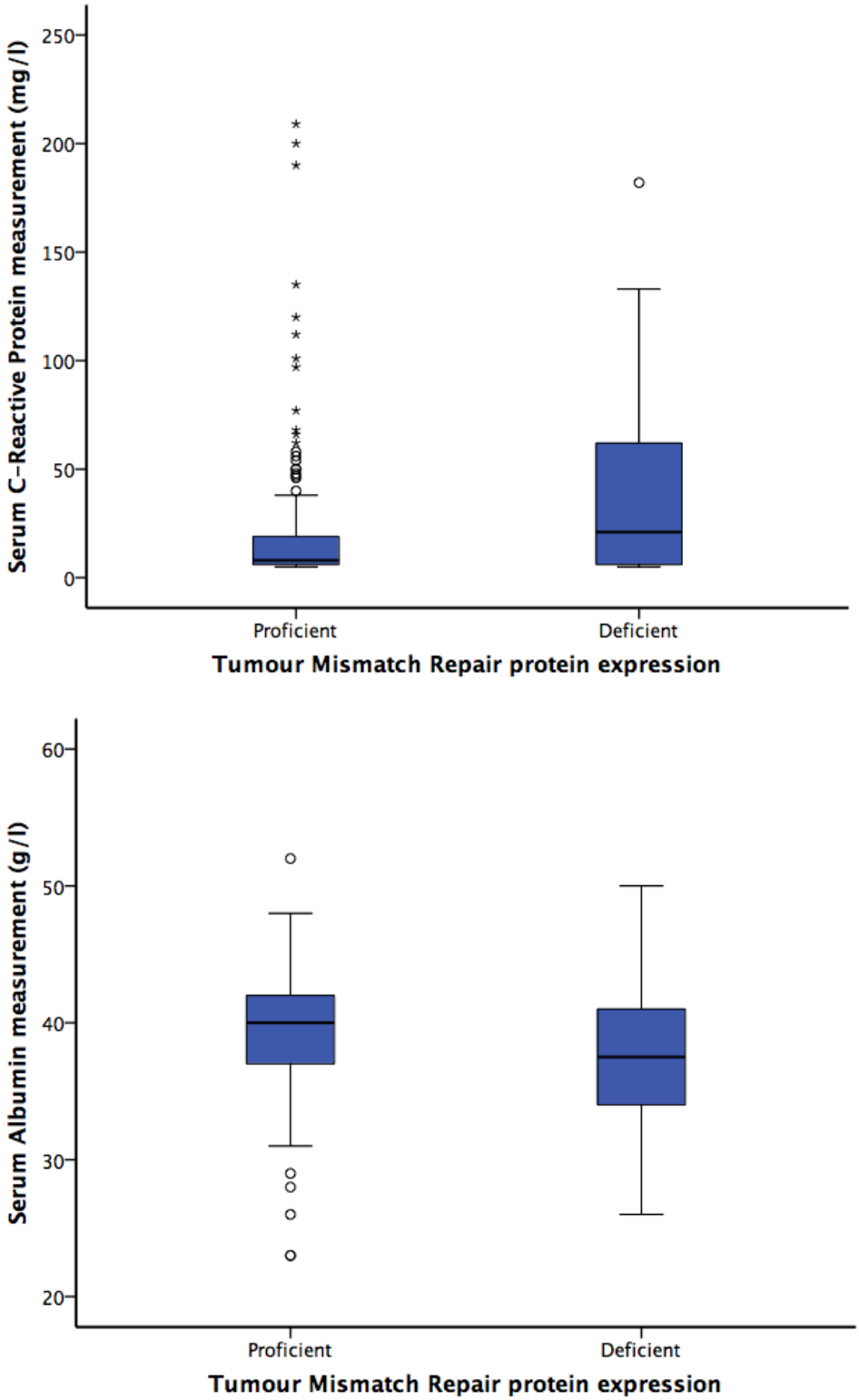


Figure 3.4: The distribution of serum CRP and albumin measurements stratified by MMR protein expression

Boxplots demonstrating serum CRP and albumin measurements stratified by tumour MMR protein expression in patients with colorectal cancer.

Table 3.3: The relationship between tumour MMR protein expression and clinicopathological factors

Clinicopathological factors	MMR Proficient	MMR Deficient	p-value
Sex			
Female	67 (43.8%)	10 (34.5%)	0.352
Male	86 (56.2%)	19 (65.5%)	
Presentation			
Elective	146 (95.4%)	28 (96.6%)	0.627*
Emergency	7 (4.6%)	1 (3.4%)	
Tumour Site			
Colon	99 (64.7%)	24 (82.8%)	0.042*
Rectum	54 (35.3%)	5 (17.2%)	
Tumour Site			
Right	48 (31.4%)	21 (72.4%)	**<0.001***
Left	51 (33.3%)	3 (10.3%)	
Rectum	54 (35.3%)	5 (17.2%)	
Differentiation			
Well-Mod	142 (92.8%)	20 (69.0%)	**<0.001**
Poor	11 (7.2%)	9 (30.0%)	
T stage			
1	3 (2.0%)	1 (3.4%)	0.141*
2	11 (7.2%)	2 (6.9%)	
3	99 (64.7%)	12 (41.4%)	
4	40 (26.1%)	14 (48.3%)	
N stage			
0	81 (52.9%)	17 (58.6%)	0.521*
1	57 (37.3%)	10 (34.5%)	
2	15 (9.8%)	2 (6.9%)	
TNM stage (simplified)			
I	9 (5.9%)	3 (10.3%)	0.633*
II	72 (47.1%)	14 (48.3%)	
III	72 (47.1%)	12 (41.4%)	
Serum CRP			
Normal	88 (57.5%)	12 (41.2%)	0.109
High	65 (42.5%)	17 (58.6%)	
Serum albumin			
Normal	135 (88.2%)	22 (75.9%)	0.076
Low	18 (11.8%)	7 (24.1%)	
Klintrup score			
Good	47 (30.9%)	10 (34.5%)	0.705
Poor	105 (69.1%)	19 (65.5%)	

Alpha following Bonferroni adjustment = <0.0032

* Fishers exact test

3.3.4 The associations between serum CRP, serum albumin, tumour MMR protein expression and survival

Patients were followed up as described in section 2.7.5.1. During follow-up there were 42 (23.1%) recurrences and 66 (36.3%) deaths. Five year recurrence-free and overall survival rates for the clinicopathological factors studied can be found in appendix 3.1.

3.3.4.1 Univariable recurrence-free survival - serum CRP expression

Serum CRP measurements were significantly higher in patients who went on to develop disease recurrence (p=0.012, Mann-Whitney U, figure 3.5). Median CRP for patients with recurrence was 15 mg/l (IQR 5 – 44.75 mg/l) compared with 7 mg/l (IQR 5 – 21.75 mg/l) in the non-recurrence group.

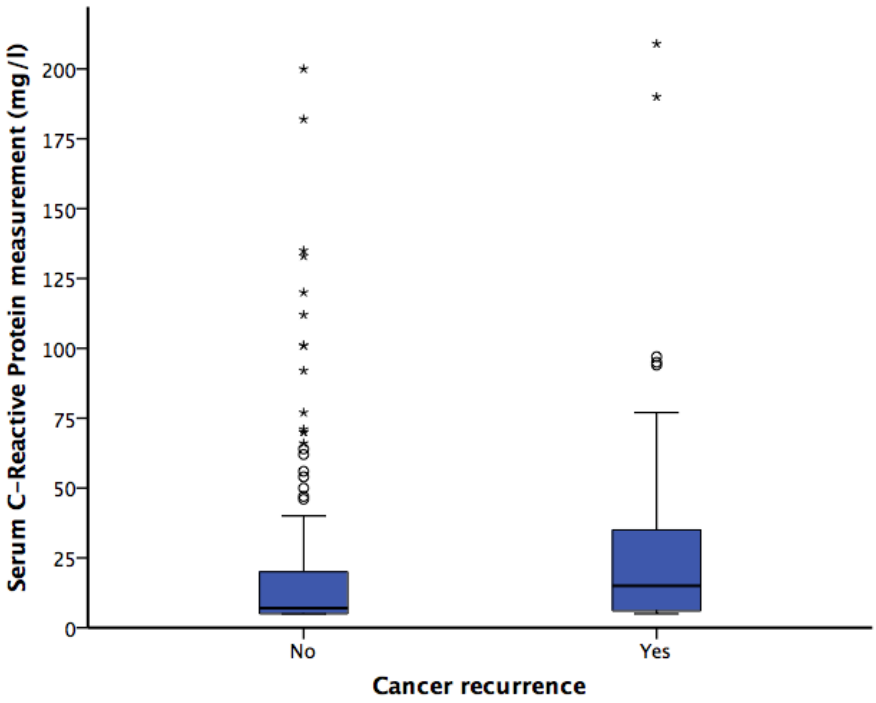
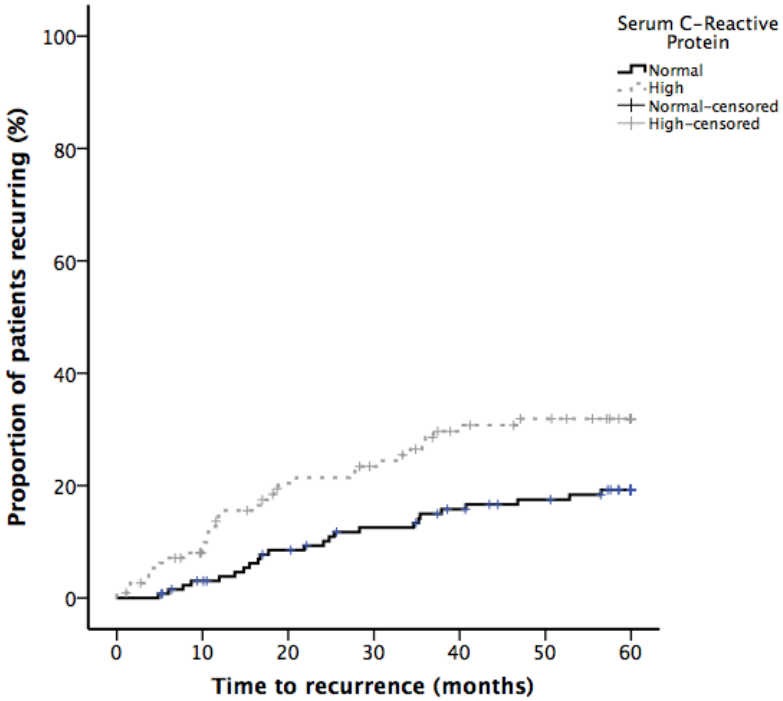


Figure 3.5: The distribution of serum CRP measurements in patients with and without disease recurrence (p=0.017)

The 5 year recurrence-free survival rate for patients with a raised serum CRP was 41.5% compared to 68.0% in patients with a normal serum CRP (p<0.001, Pearson's chi square). On logrank analysis raised serum CRP was significantly associated with poorer recurrence-free survival (p=0.007, figure 3.6). The mean survival for patients with a raised serum CRP was 45.8 months (95% CI 41.1 – 50.6) compared with 54.0 months (95% CI 51.1 – 56.9) in the normal serum CRP group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	100	93	88	82	78	74	68
High	82	68	54	50	41	37	34

Figure 3.6: The relationship between serum CRP expression and recurrence-free survival (p=0.007)

Kaplan-Meier curves demonstrating the proportion of patients recurring stratified by serum CRP expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis raised serum CRP was significantly associated with poor recurrence-free survival (Hazard Ratio (HR) 2.29 (95% CI 1.23 – 4.24), p=0.009) when dichotomised as a categorical variable. Its significance level, however, reduced when it was included as a continuous variable (HR 1.01 (95% CI 1.00 – 1.02), p=0.023). Despite the significant associations observed between serum CRP and recurrence-free survival, its predictive value remains relatively poor. When receiver-operator-characteristic analysis was performed using recurrence as the endpoint, the area under the curve (AUC) was 0.63 (95% CI 0.53 – 0.72, p=0.013) for CRP as a continuous variable and AUC 0.60 (95% CI 0.50 – 0.69, p=0.065) for CRP as a categorical variable (figure 3.7).

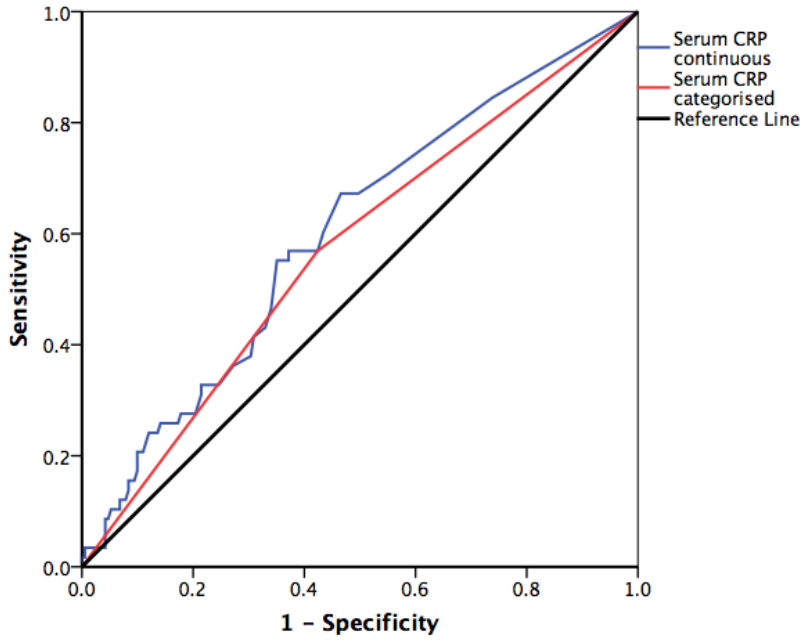


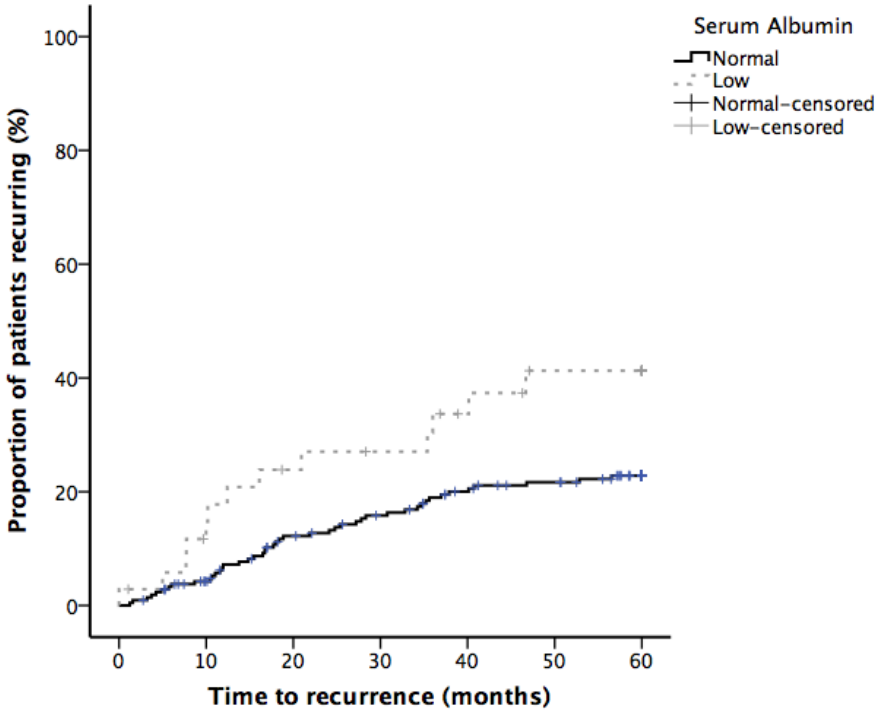
Figure 3.7: The predictive value of serum CRP in identifying patients who will develop recurrence during follow-up

Receiver-operator-characteristic curve demonstrating the predictive value of serum CRP in identifying patients who will develop disease recurrence.

3.3.4.2 Univariable recurrence-free survival - serum albumin expression

There was no statistically significant difference between the serum albumin measurements of patients who did and did not develop disease recurrence (p=0.367, Mann-Whitney U, supplementary figure 3.3, appendix 5.1). Median serum albumin was 39 g/l (IQR 33 – 45 g/l) in both the recurrence and non-recurrence groups.

The 5 year recurrence free survival rate for patients with hypoalbuminaemia was 40.0% compared to 58.6% in patients with a normal serum albumin (p=0.082, Pearson's chi square). On logrank analysis hypoalbuminaemia was associated with poor recurrence-free survival (p=0.044, figure 3.8). The mean survival for patients with hypoalbuminaemia was 43.3 months (95% CI 34.2 – 52.4) compared with 51.5 months (95% CI 48.8 – 54.3) in the normal serum albumin group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	157	142	126	118	106	101	92
High	25	19	16	14	13	10	10

Figure 3.8: The relationship between serum albumin expression and recurrence-free survival (p=0.044)

Kaplan-Meier curve demonstrating the proportion of patients recurring stratified by serum albumin expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis hypoalbuminaemia was significantly associated with poor recurrence free survival when dichotomised as a categorical variable (HR 2.10 (95% CI 1.00 – 4.38), p=0.049). It was also significantly associated with poor recurrence free survival when included as a continuous variable (HR 0.93 (95% CI 0.87 – 1.00), p=0.037). Despite the significant associations observed between serum albumin and disease recurrence, its predictive value is relatively poor. When ROC analysis was performed using recurrence as the endpoint, the AUC was 0.45 (95% CI 0.36 – 0.55, p=0.369) for albumin as a continuous variable and the AUC was 0.55 (95% CI 0.45 – 0.65, p=0.326) for albumin as a categorical variable (figure 3.9).

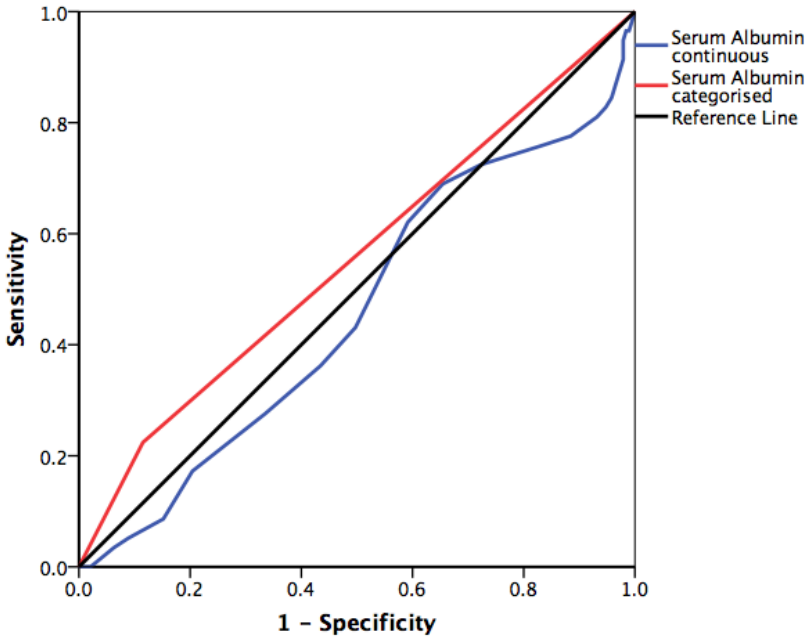
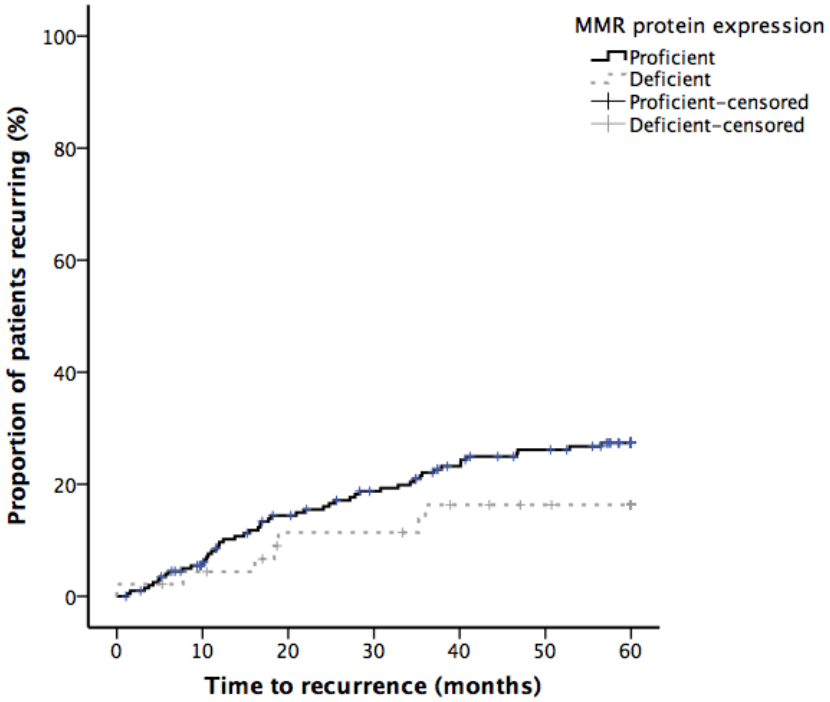


Figure 3.9: Predictive value of serum albumin in identifying patients who will develop recurrence during follow-up

Receiver-operator-characteristic curve demonstrating the predictive value of serum albumin in identifying patients who will develop disease recurrence.

3.3.4.3 Univariable recurrence-free survival - tumour MMR protein expression

Of the patients with MMR-D tumours, 3 (10.3%) developed cancer recurrence compared with 39 (25.5%) of the patients with MMR-P tumours (p=0.056, Fisher's exact test). There was no significant difference between MMR expression and recurrence-free survival on logrank analysis (p=0.085). The mean survival for MMR-P patients was 49.6 months (95% CI 46.5 – 52.6) compared with 55.0 months (95% CI 49.6 – 60.4) in the MMR-D group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
MMR-P	153	134	119	109	97	90	82
MMR-D	29	27	23	23	22	21	20

Figure 3.10: The relationship between MMR protein expression status and recurrence-free survival (p=0.085)

Kaplan-Meier curve demonstrating the proportion of patients recurring stratified by MMR protein status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis MMR-D status was not significantly associated with improved recurrence free survival (HR 0.37 (95% CI 0.12 – 1.20), p=0.098). Furthermore, MMR-D status did not significantly predict the development of recurrence with an AUC of 0.48 (95% CI 0.35 – 0.54, p=0.262)

3.3.4.4 Univariable overall survival - serum CRP expression

Serum CRP measurements were significantly higher in patients who died during follow-up (p=0.001, Mann-Whitney U, figure 3.11). Median CRP for patients who died was 14.00 mg/l (IQR 5.00 – 41.25 mg/l) compared with 6.00 mg/l (IQR 5.00 – 18.74 mg/l) in the alive group.

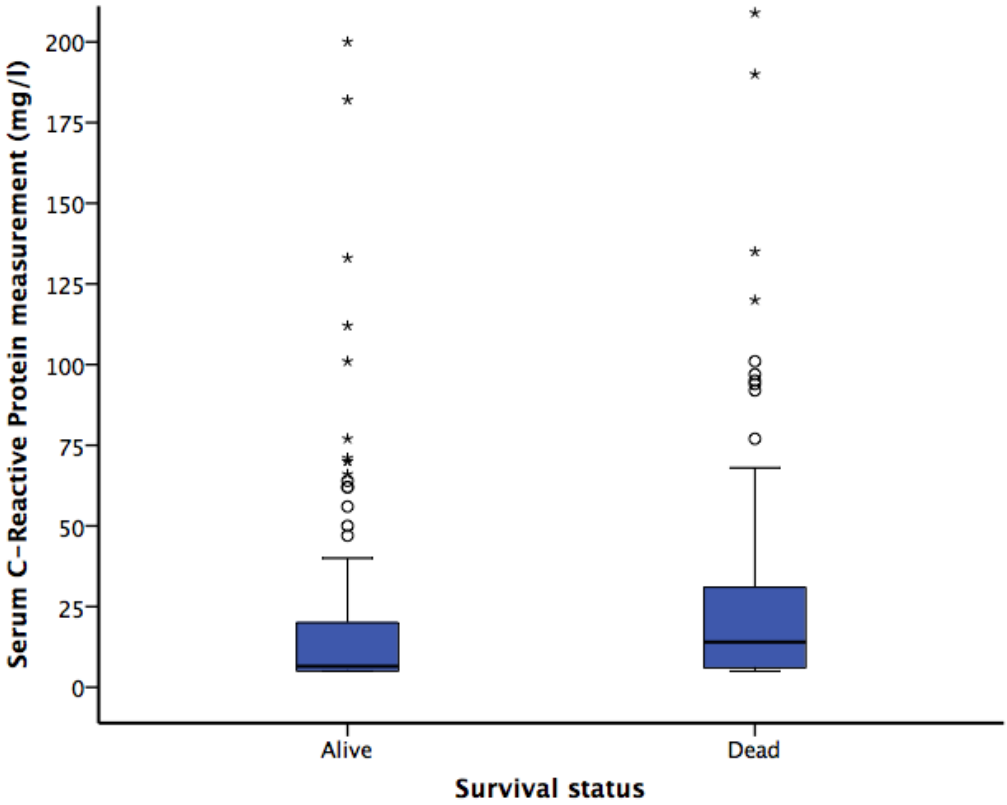
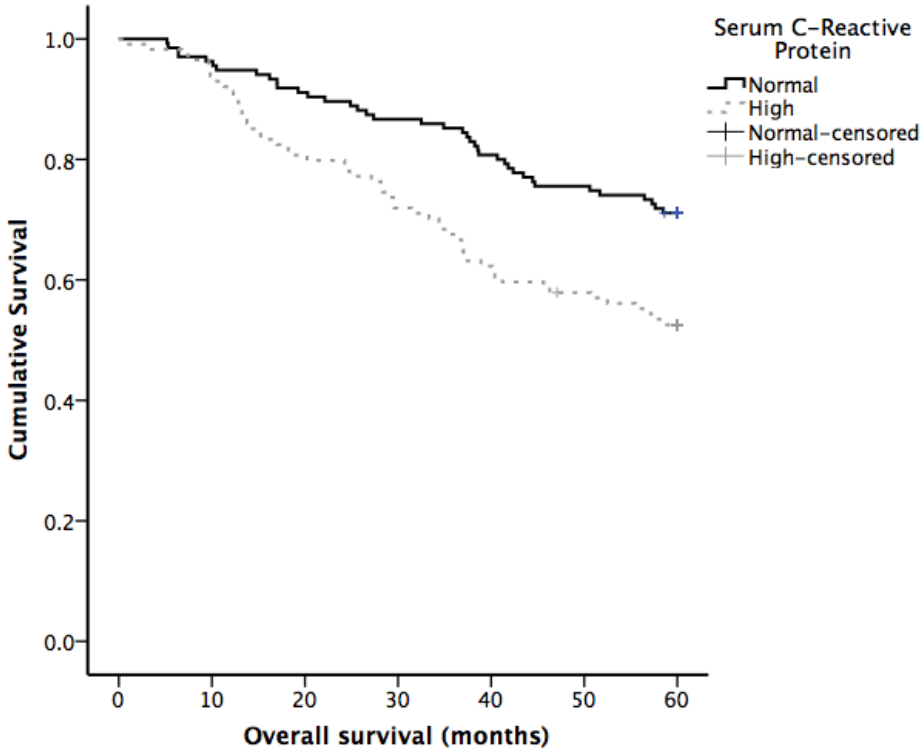


Figure 3.11: The distribution of serum CRP measurements in patients stratified by survival status (p=0.001)

The 5 year overall survival rate for patients with a raised serum CRP was 50.0% compared to 74.0% in patients with a normal serum CRP (p=0.001, Pearson's chi square). On logrank analysis raised serum CRP was significantly associated with poor overall survival (p=0.001, figure 3.12). The mean survival for patients with a raised serum CRP was 43.0 months (95% CI 38.6 – 47.4) compared with 52.5 months (95% CI 49.5 – 55.5) in the normal serum CRP group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	100	96	92	88	82	78	74
High	82	75	64	57	49	44	41

Figure 3.12: The relationship between serum CRP expression and overall survival (p=0.001)

Kaplan-Meier curve demonstrating the proportion of patients surviving stratified by serum CRP expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis raised serum CRP was significantly associated with poorer overall survival when dichotomised as a categorical variable (HR 2.31 (95% CI 1.41 – 3.79), p=0.001); however, its significance reduced when it was included as a continuous variable (HR 1.01 (95% CI 1.00 – 1.01), p=0.014). Despite the significant associations observed between serum CRP and poor overall survival, its predictive value is relatively poor. When ROC analysis was performed using death as the endpoint, the AUC was 0.65 (95% CI 0.56 – 0.73, p=0.001) for CRP as a categorical variable and AUC 0.62 (95% CI 0.54 – 0.71, p=0.006) for CRP as a continuous variable (figure 3.13).

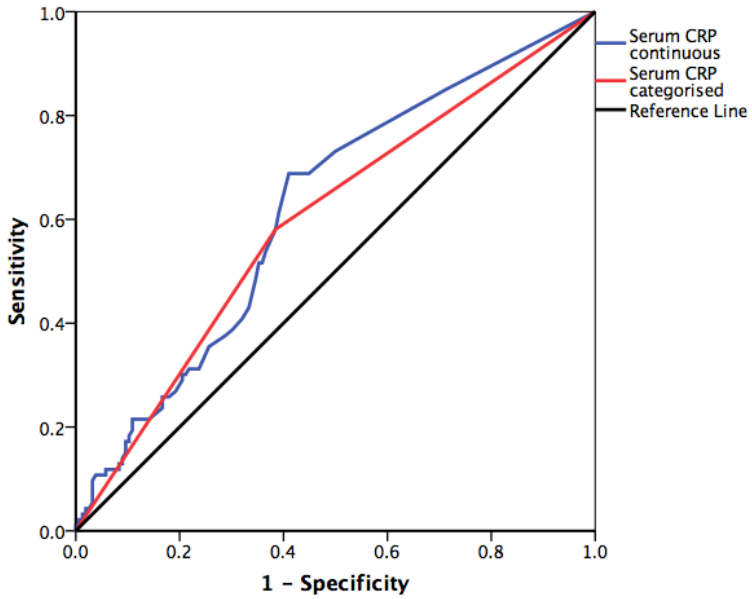


Figure 3.13: Predictive value of CRP in identifying patients who will die during the follow up period

Receiver-operator-characteristic curve demonstrating the predictive value of CRP in identifying patients who will die during follow-up.

3.3.4.5 Univariable overall survival - serum albumin expression

There was no statistically significant between the serum albumin measurements of patients who died and those that survived to 5 years (p=0.162, Mann-Whitney U, supplementary figure 3.4, appendix 5.1). Median serum albumin measurements were 40 g/l (IQR 34 – 46 g/l) in the alive group compared to 39 g/l (IQR 33 – 45 g/l) in patients who died.

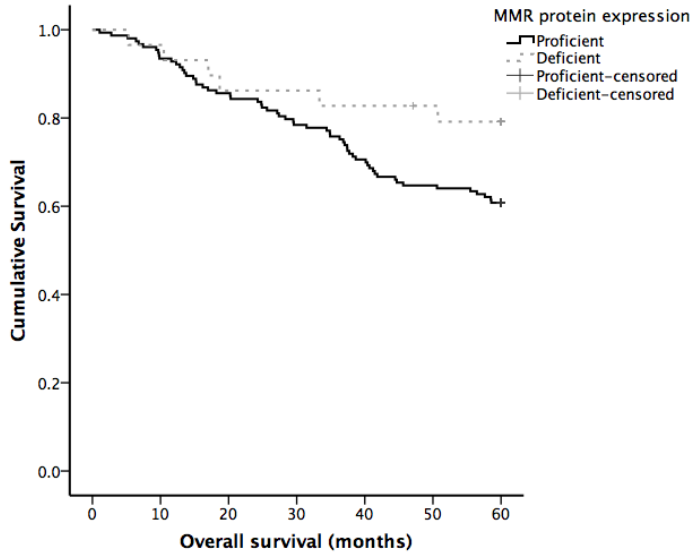
The 5 year overall survival rate for patients with hypoalbuminaemia was 48.0% compared to 65.6% in patients with a normal serum albumin (p=0.090, Pearson's chi square). On logrank analysis hypoalbuminaemia was not associated with poor overall survival (p=0.110). The mean survival for patients with hypoalbuminaemia was 41.9 months (95% CI 33.7 – 50.0) compared with 49.2 months (95% CI 46.5 – 52.0) in the normal serum albumin group (supplementary figure 3.5, appendix 5.1).

On Cox univariable regression analysis hypoalbuminaemia was not significantly associated with poor overall survival when dichotomised as a categorical variable (HR 1.66 (95% CI 0.89 – 3.10), p=0.114), however, it was significantly associated with survival when included as a continuous variable (HR 0.94 (95% CI 0.90 – 0.99), p=0.027). In terms of survival prediction, when ROC analysis was performed using death as the endpoint, the AUC was 0.44 (95% CI 0.35 – 0.52, p=0.164) for albumin as a continuous variable and an

AUC of 0.54 (95% CI 0.45 – 0.62, p=0.435) for albumin as a categorical variable (supplementary figure 3.6, appendix 5.1).

3.3.4.6 Univariable overall survival - Tumour MMR expression

Of the patients with MMR-D tumours, 6 (20.7%) died during the follow up period compared with 60 (39.2%) patients in the MMR-P tumour group (p=0.042, Fisher's exact test). There was no significant difference between tumour MMR expression status and overall survival on logrank analysis (p=0.083, figure 3.14). The mean survival for MMR-P patients was 47.4 months (95% CI 44.5 – 50.4) compared with 52.2 months (95% CI 46.2 – 58.3) in the MMR-D group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
MMR-P	153	143	131	120	108	99	93
MMR-D	29	28	25	25	24	23	22

Figure 3.14: The relationship between MMR protein expression and overall survival (p=0.083)

Kaplan-Meier curve demonstrating the proportion of patients surviving stratified by MMR protein status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis MMR-D status was not significantly associated with improved overall survival (HR 0.48 (95% CI 0.21 – 1.12), p=0.090). Furthermore, MMR-D status did not significantly predict death during follow-up with an AUC of 0.43 (95% CI 0.36 – 0.53, p=0.229)

3.3.4.7 Multivariable recurrence-free survival

Proportionality assumptions testing

All the studied variables underwent Cox proportional hazards assumption testing as described in the statistical methodology section 2.7.8.7.

Cox proportional hazards regression analysis

All covariates with a significance level of $p<0.1$ were included in the multivariable model. Terms were removed if their respective p was >0.05. On univariable analysis, emergency presentation (p=0.045), advancing T-stage (p=0.046), advancing N-stage (p=0.003), higher TNM stage (p=0.003), raised serum CRP (p=0.009), hypoalbuminaemia (p=0.049) and tumour MMR-D status (p=0.098) had a p-value <0.1 and were therefore included in the multivariable Cox proportional hazards regression model.

Raised serum CRP (HR 2.52 (95% CI 1.21 - 5.25), p=0.014), hypoalbuminaemia (HR 2.46 (95% CI 1.07 - 5.68), p=0.034) and MMR-D status (HR 2.52 (95% CI 0.06 – 0.64), p=0.007) were significantly and independently associated with recurrence-free survival (table 3.4).

Table 3.4: The relationship between clinicopathological factors, SIR, LIR and MMR status and recurrence free survival: multivariable analysis

	Univariable analysis			Multivariable analysis		
	Coefficient	p-value	Hazard ratio (95% CI)	Coefficient	p-value	Hazard ratio (95% CI)
Age	-0.007	0.605	0.99 (0.97 - 1.02)			
Sex						
Female			1			
Male	0.211	0.511	0.81 (043 – 1.52)			
Presentation						
Elective			1			
Emergency	1.052	0.045	2.86 (1.02 – 8.02)		0.302	
Tumour site						
Right	0.000	0.454	1			
Left	-0.089		0.92 (0.46 – 1.84)			
Rectum	0.485		0.62 (0.28 – 1.33)			
T stage						
1	0.000	0.046	1		0.072	
2	9.082		8792.34 (0.00 – 3.19e80)			
3	8.813		6720.81 (0.00 – 2.43e80)			
4	9.712		16521.90 (0.00 – 5.98e80)			
N stage						
0	0.000	0.003	1		0.441	
1	1.124		3.08 (1.57 – 6.05)			
2	1.169		3.22 (1.15 – 9.05)			
TNM stage						
I	0.000	0.003	1		0.060	
II	0.232		0.79 (0.18 – 3.58)			
III	0.932		2.54 (0.61 – 10.65)			
Differentiation						
Well/Moderate			1			
Poor	0.314	0.510	1.37 (0.54 – 3.49)			
Serum CRP						
Normal			1			1
High	0.826	0.009	2.29 (1.23 – 4.24)	0.922	0.014	2.52 (1.21 – 5.25)
Serum albumin						
Normal			1			1
Low	0.740	0.049	2.10 (1.00 – 4.38)	0.901	0.034	2.46 (1.07 – 5.68)
Klintrup score						
High			1			
Low	0.515	0.155	1.67 (0.82 – 3.40)			
MMR status						
MMR-P			1			1
MMR-D	-0.991	0.098	0.37 (0.12 – 1.20)	-1.657	0.007	0.19 (0.06 – 0.64)

3.3.4.8 Multivariable overall survival

Proportionality assumptions testing

All the studied variables underwent Cox proportional hazards assumption testing as described in the statistical methodology section 2.7.8.7.

Cox proportional hazards regression analysis

All covariates with a significance level of $p<0.1$ were included in the multivariable model. Terms were removed if their respective p was >0.05. On univariable analysis, advancing age (p=0.025), advancing N-stage (p=0.002), higher TNM stage (p=0.026), raised serum CRP (p=0.001), poor Klintrup score (p=0.008) and tumour MMR-D status (p=0.090) had a p-value <0.1 and were therefore included in the multivariable Cox proportional hazards regression model.

On multivariable analysis, raised serum CRP (HR 2.40 (95% CI 1.44 – 53.99), p=0.001), advancing age (HR 1.02 (95% CI 1.00 – 1.05), p=0.045), poor Klintrup score (HR 2.04 (95% CI 1.08 – 3.85), p=0.027) and MMR-D status (HR 0.41 (95% CI 0.18 – 0.97), p=0.043) were independently and significantly associated with poor overall survival following (table 3.5).

Table 3.5: The relationship between clinicopathological factors, SIR, LIR and MMR status and survival: multivariable analysis

	Univariable analysis			Multivariable analysis		
	Coefficient	p-value	Hazard ratio (95% CI)	Coefficient	p-value	Hazard ratio (95% CI)
Age	0.026	0.025	1.03 (1.00 - 1.05)	0.024	0.045	1.02 (1.00 – 1.05)
Sex						
Female			1			
Male	-0.206	0.404	0.81 (0.50 – 1.32)			
Presentation						
Elective			1			
Emergency	0.531	0.304	1.70 (0.62 – 4.68)			
Tumour site						
Right	0.000	0.788	1			
Left	-0.159		0.85 (0.47 – 1.56)			
Rectum	0.053		1.05 (0.60 – 1.86)			
T stage						
1	0.000	0.134	1			
2	8.573		5286.13 (0.00 – 6.00e63)			
3	8.930		7554.37 (0.00 – 8.54e63)			
4	9.474		13019.00 (0.00 – 1.47e64)			
N stage						
0	0.000	0.002	1		0.053	
1	0.500		1.65 (0.65 – 2.80)			
2	1.250		3.51 (1.74 – 7.08)			
TNM stage						
I	0.000	0.026	1		0.903	
II	0.600		1.82 (0.43 – 7.70)			
III	1.203		2.54 (0.80 – 13.79)			
Differentiation						
Well/Moderate			1			
Poor	0.444	0.216	1.56 (0.77 – 3.15)			
Serum CRP						
Normal			1			1
High	0.838	0.001	2.31 (1.41 – 3.79)	0.778	0.001	2.40 (1.44 – 3.99)
Serum albumin						
Normal			1			
Low	0.506	0.114	1.56 (0.87 – 3.10)			
Klintrup score						
High			1			1
Low	-0.853	0.008	2.35 (1.25 – 4.40)	0.737	0.027	2.04 (1.08 – 3.85)
MMR status						
MMR-P			1			1
MMR-D	-0.727	0.090	0.48 (0.21 – 1.12)	-0.882	0.043	0.41 (0.18 – 0.97)

3.4 Validation cohort

3.4.1 Systemic inflammation quantification

3.4.1.1 Serum CRP expression

Serum CRP was measured using ELISA and reported in mg/l as described in section 3.3.1.1. Figure 3.15 demonstrates that the expression of serum CRP follows an exponential distribution (histogram, figure 3.15) with measurements >75 mg/l considered outliers. Serum measurements ranged from 0.90 – 460.00 mg/l with a median of 10.00 mg/l (IQR 0.90 – 35.00 mg/l) (supplementary figure 3.7, appendix 5.1). Using the dichotomisation thresholds described by McMillan et al, 49.3% of patients were considered high expressers (>10 mg/l).

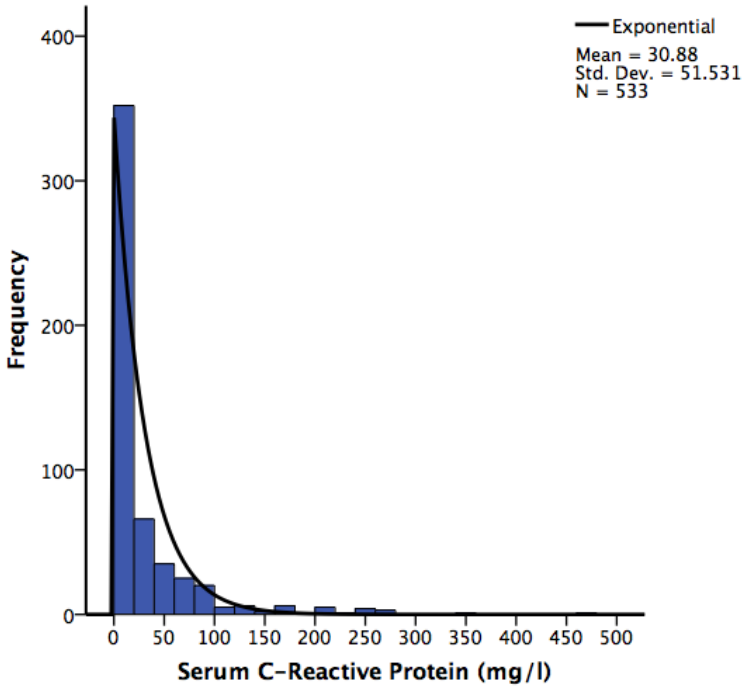


Figure 3.15: Histogram demonstrating the distribution of serum CRP in patients with colorectal cancer

3.4.1.2 Serum albumin expression

Serum albumin was measured using ELISA and reported in g/l. Figure 3.16 demonstrates that the expression of serum albumin does not precisely follow a normal distribution (histogram, figure 3.16), which is supported by a Shapiro-Wilk statistic of 0.955, df 533, $p<0.001$. The measurements ranged from 12 – 51 g/l with a median of 39 g/l (IQR 30 - 48 g/l) (supplementary figure 3.8, appendix 5.1). Using the dichotomisation thresholds by McMillan et al, 26.6% of patients were considered hypoalbuminaemic (<35 g/l).

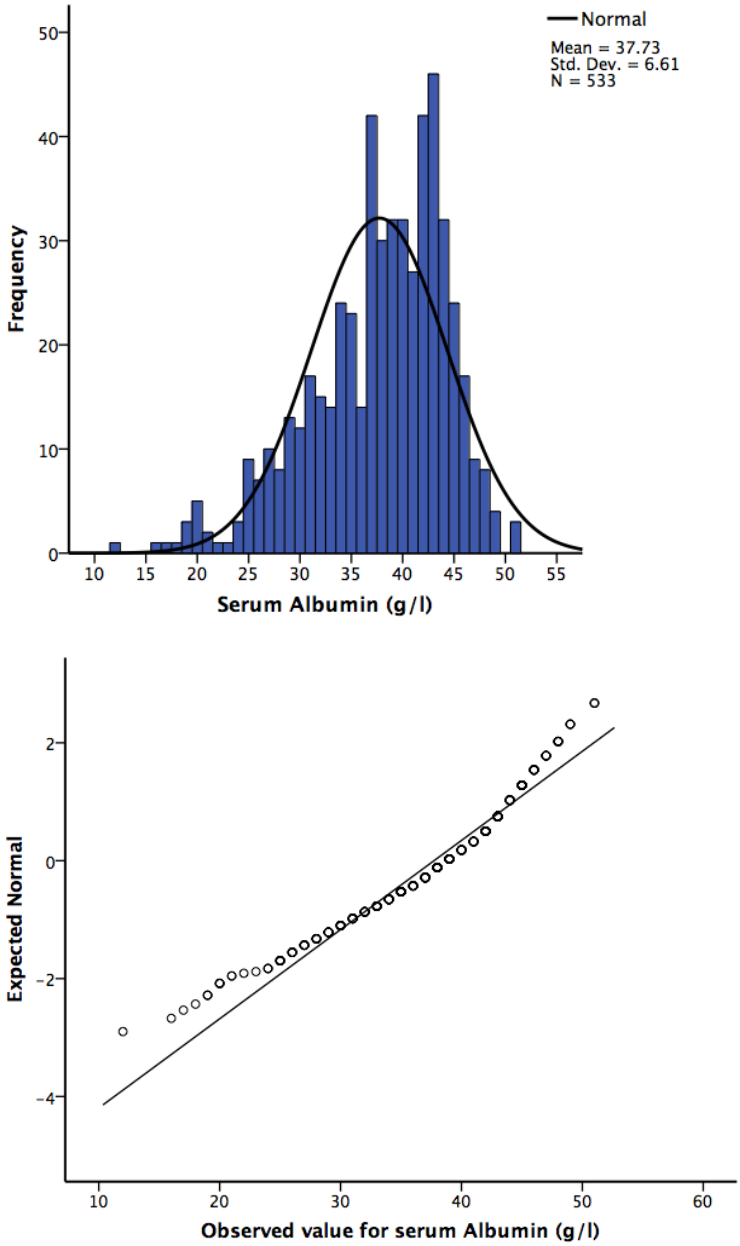


Figure 3.16: Distribution of measurements of serum albumin in patients with colorectal cancer

Histogram demonstrating the distribution of serum albumin measurements. Q-Q Plot of Serum albumin measurements.

3.4.2 MMR protein status evaluation and microsatellite instability

Using immunohistochemistry, MMR protein expression was evaluated by assessing the expression of the MMR proteins; MLH1, MSH2, PMS2, MSH6. NEQUAS reporting guidelines were used when assessing expression. Tumours expressed MLH1 in 88% of cases, MSH2 in 94% of cases, PMS2 in 86% of cases and MSH6 in 93% of cases. Using NEQUS reporting guidelines n=107 (15.8%) of the resected colorectal cancers were considered MMR deficient. Following MSI analysis, 85 (13%) of the 677 patients within

the cohort had microsatellite unstable tumours. Of these 85 patients with MSI tumours, 79 (92.9%) were MMR deficient. In addition, 28 (4.7%) of the 592 MSS tumours were also MMR deficient (p<0.001, chi squared analysis).

3.4.3 The association of MSI status and clinicopathological factors

Patients with MSI tumours had higher levels of serum CRP with a median of 20.00 mg/l (IQR 1.00 – 65.50 mg/l) compared with a median of 9.00 mg/l (IQR 0.90 – 31.00 mg/l) in patients with CI tumours (p<0.001, Mann-Whitney U, figure 3.17). This association was also supported by chi-squared analysis when the variables were analysed as categorical data (p=0.003, Bonferroni adjustment p<0.0032). On chi-squared analysis of categorical variables, right-sided tumour location (p<0.001), poor differentiation (p<0.001) and MMR-D status (p<0.001) were significantly associated with MSI tumours following Bonferroni adjustment (table 3.5). When serum albumin was analysed as a continuous variable, there was no statistically significant difference between serum measurements and MSI status (p=0.156, Mann-Whitney U, supplementary figure 3.9, appendix 5.1).

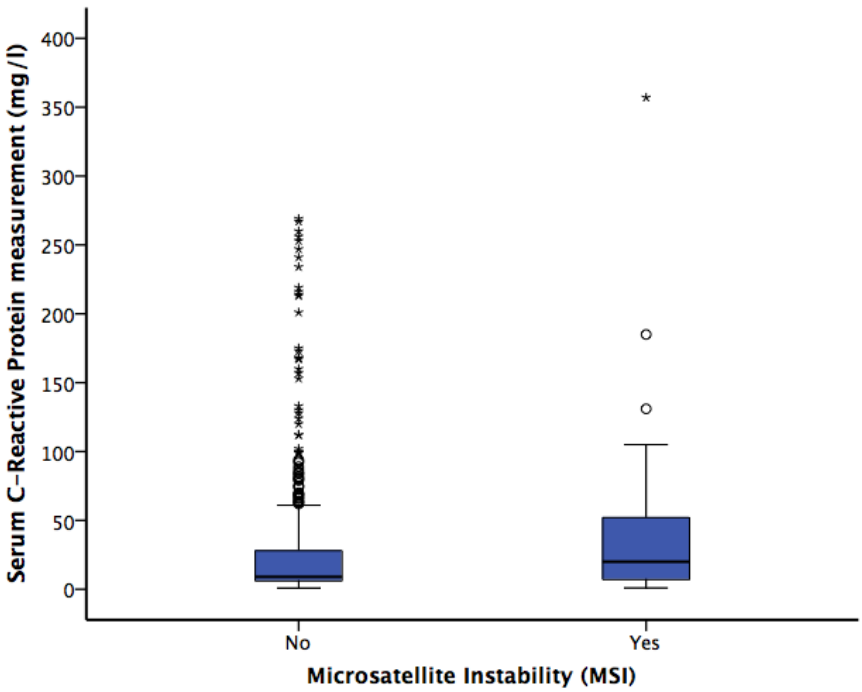


Figure 3.17: The distribution of serum CRP measurements in patients stratified by MSI status (p<0.001)

Boxplots demonstrating the difference in serum CRP measurements stratified by tumour MSI status.

Table 3.6: The relationships between tumour MSI status and categorical clinicopathological factors

Clinicopathological factors	Chromosomal Instability (CI)	Microsatellite Instability (MSI)	p-value
Age	73 (IQR 57 – 89)	71 (IQR 55 – 87)	0.908
Sex			
Female	277 (46.8%)	55 (64.7%)	0.002
Male	315 (53.2%)	30 (35.3%)	
Presentation			
Elective	413 (69.8%)	63 (74.1%)	0.411
Emergency	179 (30.2%)	22 (25.9%)	
Tumour Site			
Colon	456 (77.0%)	78 (91.8%)	**0.001***
Rectum	136 (23.0%)	7 (8.2%)	
Tumour Site			
Right	233 (39.4%)	69 (81.2%)	**<0.001**
Left	223 (37.7%)	10 (11.8%)	
Rectum	136 (23.0%)	6 (7.1%)	
Differentiation			
Well-Mod	544 (91.9%)	67 (78.8%)	<0.001
Poor	48 (8.1%)	18 (21.2%)	
T stage			
1	23 (3.9%)	4 (4.7%)	0.557*
2	80 (13.5%)	11 (12.9%)	
3	321 (54.2%)	40 (47.1%)	
4	168 (28.4%)	30 (35.3%)	
N stage			
0	375 (63.3%)	64 (75.3%)	
1	150 (25.3%)	16 (18.8%)	0.083*
2	67 (11.3%)	5 (5.9%)	
TNM stage (simplified)			
I	90 (15.2%)	14 (16.5%)	
II	278 (47.0%)	50 (58.8%)	0.056
III	224 (37.8%)	21 (24.7%)	
Serum CRP			
Normal	248 (53.1%)	22 (33.3%)	0.003
High	219 (46.9%)	44 (66.7%)	
Serum albumin			
Normal	369 (74.4%)	44 (65.7%)	0.130
Low	127 (25.6%)	23 (34.3%)	
Klintrup score			
Good	173 (29.2%)	33 (38.8%)	0.072
Poor	419 (70.8%)	52 (61.2%)	
MMR Status			
Proficient	564 (95.3%)	6 (7.1%)	<0.001
Deficient	28 (4.7%)	79 (92.9%)	

Bonferoni adjustment = <0.0032

* Fishers exact test

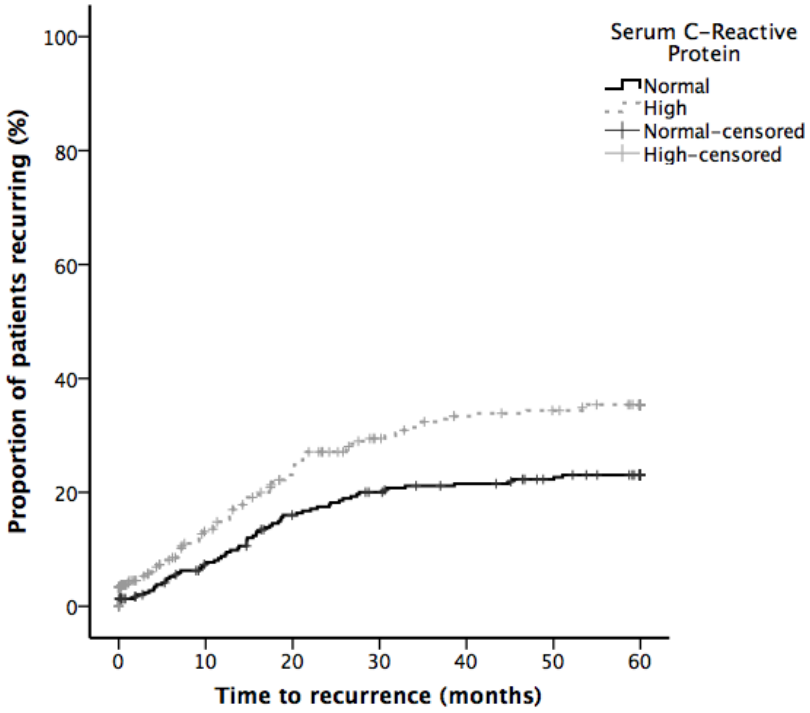
3.4.4 The associations of serum CRP, serum albumin, tumour MSI status and survival

During follow-up there were 150 (22.2%) recurrences and 260 (38.4%) deaths. Five year recurrence-free and overall survival rates for the clinicopathological factors studied can be found in appendix 3.2. Based on a 15% difference in 5 year survival with an α=0.05 and β=0.2 the number of patient required was n=338. For a multivariable model containing at least 13 variables a minimum of 130 events are required.

3.4.4.1 Univariable recurrence-free survival - serum CRP expression

Serum CRP measurements were not significantly different in patients who went on to develop disease recurrence (p=0.112, Mann-Whitney U, supplementary figure 3.10, appendix 5.1). Median CRP for patients with recurrence was 16.00 mg/l (IQR 1.00 – 61.75 mg/l) compared with 7.20 mg/l (IQR 0.90 – 24.20 mg/l) in the non-recurrence group.

The 5 year recurrence free survival rate for patients with a raised serum CRP was 43.0% compared to 64.8% in patients with a normal serum CRP (p<0.001, Pearson's chi square). On logrank analysis, raised serum CRP was significantly associated with poor recurrence free survival (p=0.013, figure 3.18). The mean survival for patients with a raised serum CRP was 46.8 months (95% CI 44.1 – 49.6) compared with 51.1 months (95% CI 48.8 – 53.3) in the normal serum CRP group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	270	236	212	200	192	184	175
High	263	194	161	137	126	122	113

Figure 3.18: The relationship between serum CRP expression and recurrence-free survival in patients with colorectal cancer (p=0.013)

Kaplan-Meier curve demonstrating the proportion of patients recurring stratified by serum CRP expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis raised serum CRP was significantly associated with poor recurrence free survival when dichotomised as a categorical variable (HR 1.58 (95% CI 1.10 – 2.27), p=0.013). The significance of the hazard ratio reduced when it was included as a continuous variable (HR 1.00 (95% CI 1.00 – 1.01), p=0.022). Despite the significant associations observed between serum CRP and disease recurrence, its predictive value remains poor. When ROC analysis was performed using recurrence as the endpoint, the AUC was 0.55 (95% CI 0.49 – 0.61, p=0.151) for CRP as a continuous variable and AUC 0.54 (95% CI 0.48 – 0.60, p=0.221) for CRP as a categorical variable (supplementary figure 3.11, appendix 5.1).

3.4.4.2 Univariable recurrence-free survival - serum albumin

There was no statistically significant difference between the serum albumin measurements of patients who did and did not develop disease recurrence during follow-up (p=0.061, Mann-Whitney U, figure 3.19). Median serum albumin measurements were 39 g/l (IQR 30 – 48 g/l) in the non-recurrence group compared to 38 g/l (IQR 29 – 47 g/l) in the recurrence group.

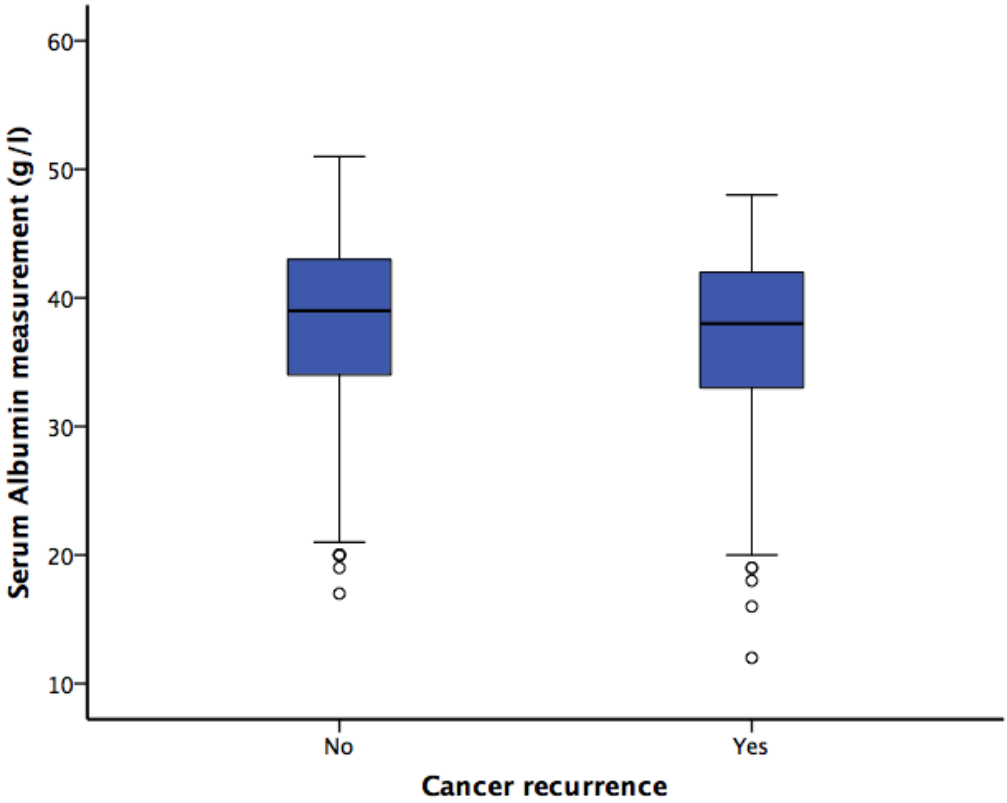
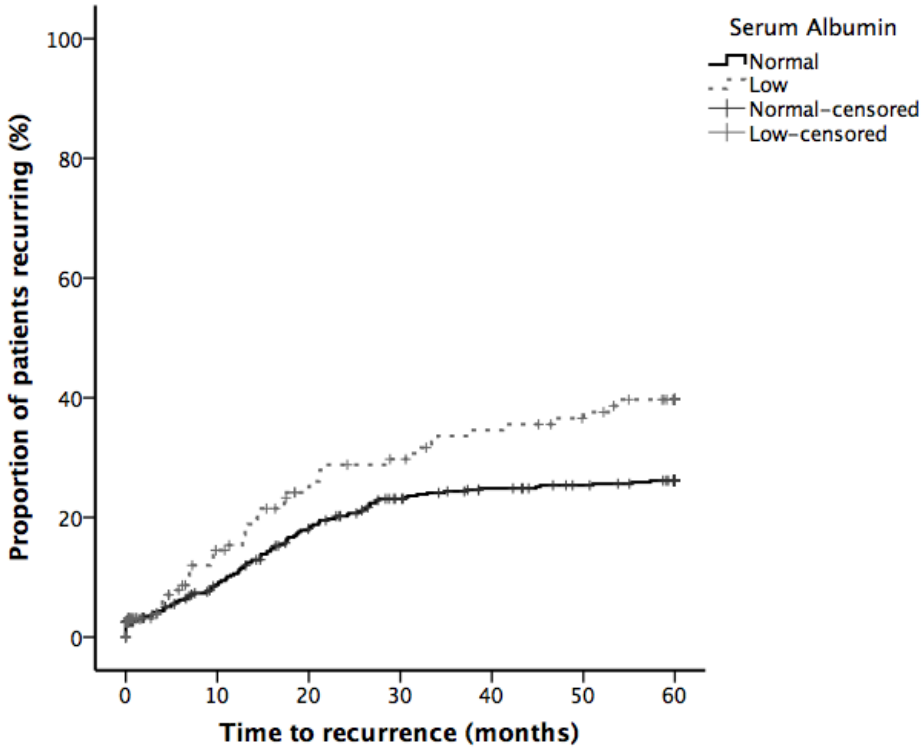


Figure 3.19: The distribution of serum albumin measurements in patients with and without cancer recurrence (p=0.061)

The 5 year recurrence free survival rate for patients with hypoalbuminaemia was 34.0% compared to 61.7% in patients with a normal serum albumin (p<0.001, Pearson's chi square). On logrank analysis hypoalbuminaemia was associated with poor recurrence-free survival (p=0.010, figure 3.20). The mean survival for patients with hypoalbuminaemia was 45.8 months (95% CI 41.8 – 49.7) compared with 50.3 months (95% CI 48.4 – 52.1) in the normal serum albumin group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	413	361	319	288	274	265	255
Low	150	98	79	72	66	61	51

Figure 3.20: The relationship between serum albumin expression and recurrence-free survival (p<0.001)

Kaplan-Meier curve demonstrating the proportion of patients recurring stratified by serum albumin expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis hypoalbuminaemia was significantly associated with poor recurrence-free survival when dichotomised as a categorical variable (HR 1.64 (95% CI 1.12 – 2.40), p=0.011). The significance of the hazard ratio increased when it was included as a continuous variable (HR 0.95 (95% CI 0.93 – 0.97), p<0.001). Despite the significant associations observed between serum albumin and disease recurrence, its predictive value is relatively poor. When ROC analysis was performed using recurrence as the endpoint, the AUC was 0.45 (95% CI 0.39 – 0.50, p=0.062) for albumin as a continuous variable and AUC 0.52 (95% CI 0.47 – 0.58, p=0.438) for albumin as a categorical variable (figure 3.21).

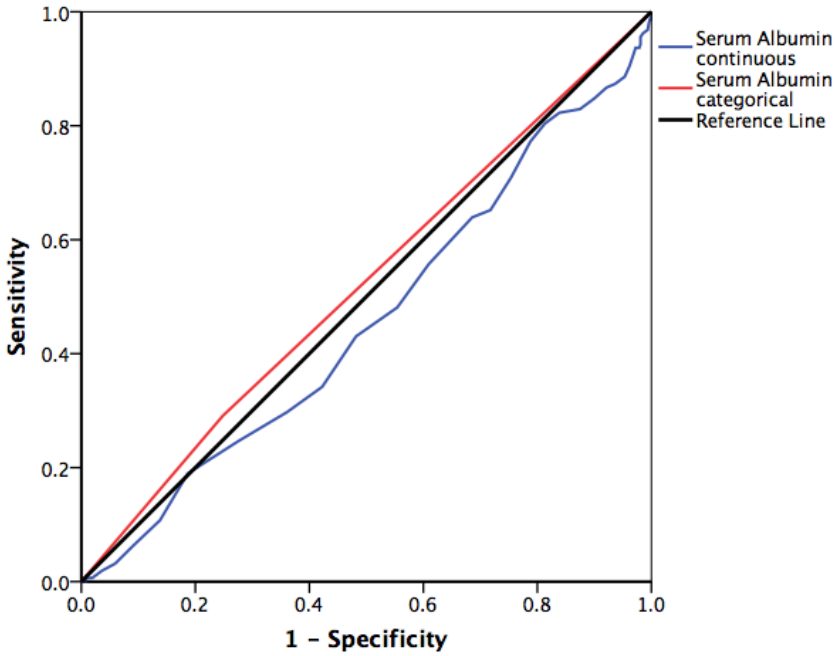
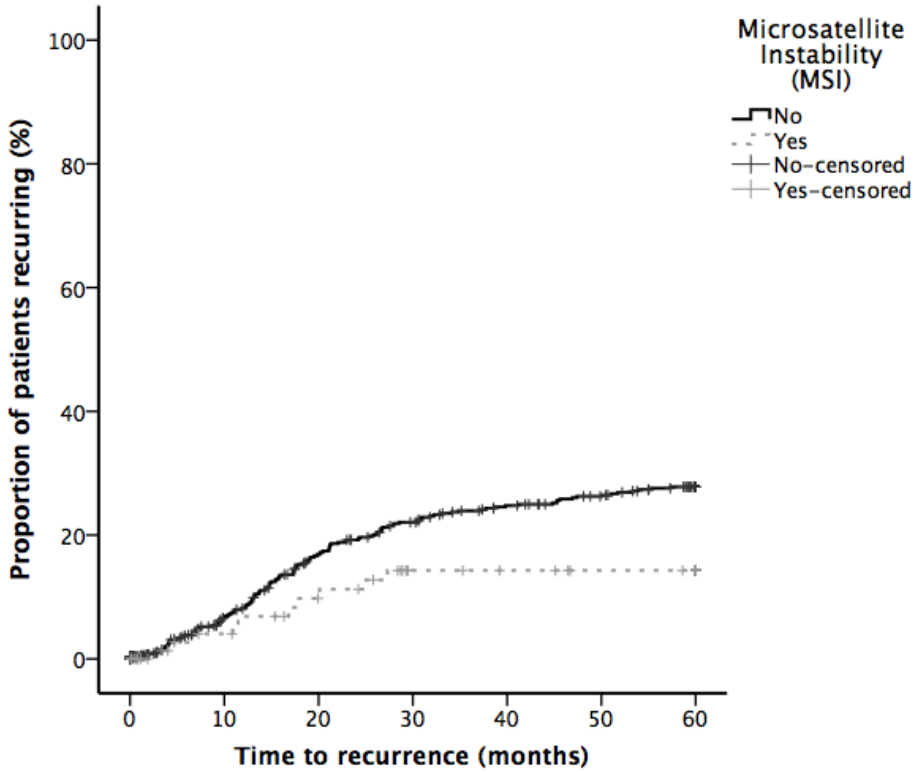


Figure 3.21: Predictive value of serum albumin in identifying patients who will develop cancer recurrence

Receiver-operator-characteristic curve demonstrating the predictive value of serum albumin in identifying patients who will develop cancer recurrence during follow-up.

3.4.4.3 Univariable recurrence-free survival - tumour MSI status

Of the patients with MSI tumours, 10 (11.8%) developed cancer recurrence compared with 140 (23.6%) of the patients with Chromosomal Instability (CI) tumours (p=0.012, Fisher's exact test). Despite this, the 5 year recurrence-free survival rate for patients with MSI tumours was 51.8% compared to 53.5% (p=0.758, Pearson's chi square) compared to patients with CI tumours. On logrank analysis, MSI status was associated with improved recurrence free survival (p=0.032, figure 3.22). The mean survival for patients with MSI tumours was 53.5 months (95% CI 49.8 – 57.3) compared with 48.9 months (95% CI 47.2 – 50.5) in the CI tumour group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
CI	592	479	415	382	357	341	317
MSI	85	69	61	50	48	45	44

Figure 3.22: The relationship between tumour MSI status and recurrence-free survival (p=0.032)

Kaplan-Meier curve demonstrating the proportion of patients recurring stratified by tumour MSI status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis, MSI tumours were significantly associated with improved recurrence free survival (HR 0.71 (95% CI 0.51 – 0.98), p=0.035), however, MSI status did not significantly predict the development of recurrence with an AUC of 0.46 (95% CI 0.41 – 0.51, p=0.157)

3.4.4.4 Univariable overall survival - serum CRP expression

Serum CRP measurements were significantly higher in patients who died during the follow up period (p<0.001, Mann-Whitney U, figure 3.23). The median CRP for patients who died was 16.00 mg/l (IQR 1.00 – 61.75 mg/l) compared with 7.20 mg/l (IQR 0.90 – 24.20 mg/l) in the alive group.

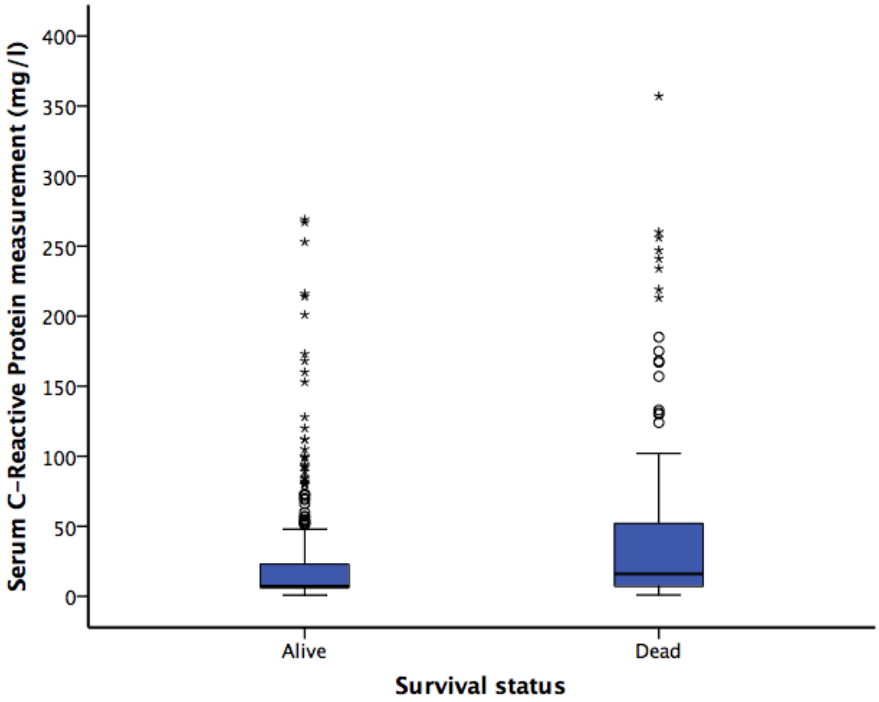
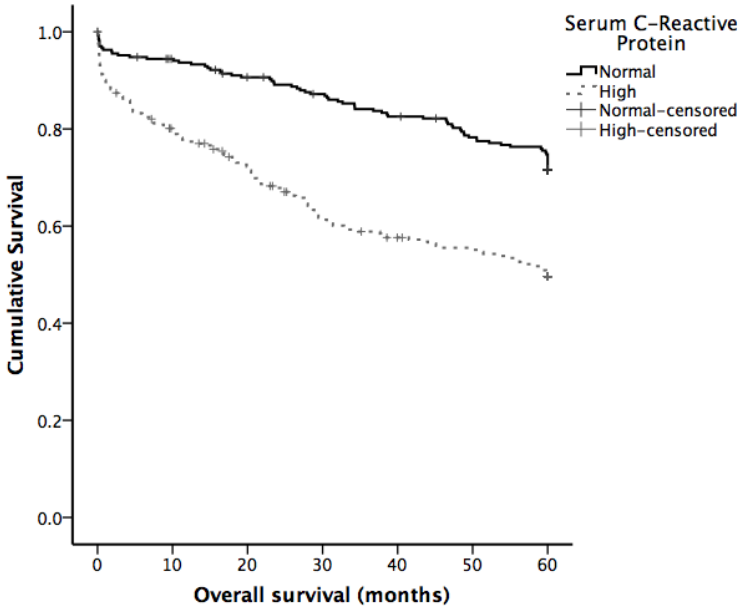


Figure 3.23: The distribution of serum CRP measurements in patients stratified by survival status (p<0.001)

The 5 year overall survival rate for patients with a raised serum CRP was 45.2% compared to 70.7% in patients with a normal serum CRP (p<0.001, Pearson's chi square). On logrank analysis, raised serum CRP was significantly associated with poor overall survival (p<0.001, figure 3.24). The mean survival for patients with a raised serum CRP was 39.6 months (95% CI 36.7 – 42.6) compared with 51.9 months (95% CI 49.9 – 54.0) in the normal serum CRP group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	270	250	237	226	214	201	192
High	263	205	180	151	138	131	119

Figure 3.24: The relationship between serum CRP expression and overall survival in patients with colorectal cancer (p<0.001)

Kaplan-Meier curve demonstrating the proportion of patients surviving stratified by serum CRP expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis raised serum CRP was significantly associated with poor overall survival when dichotomised as a categorical variable (HR 2.27 (95% CI 1.70 – 3.02), p<0.001). The hazard ratio maintained statistical significance when it was included as a continuous variable (HR 1.01 95% CI 1.00 – 1.01, p<0.001). The results of the Kaplan-Meier plot, however, suggest that serum CRP violates the proportionality assumption of the Cox proportional hazards model. There appears to be a greater proportion of events in the high expression group near the origin of the curve. The subsequent gradient associated with these events does not hold throughout the follow up phase and suggests that the HR is not constant thus violating proportionality. When assessing the log(-log(survival)) plot (figure 3.25), categorical serum CRP demonstrates parallel curves, however, there is evidence of time dependency when an interaction term between serum CRP and log(time) was placed in a Cox proportional hazard model (HR 0.98 (95% CI 0.96 – 0.99); p=0.002) with an associated adjustment of the HR for categorical CRP (HR 4.22 (95% CI 2.56 – 6.96); p<0.001) (table 3.7).

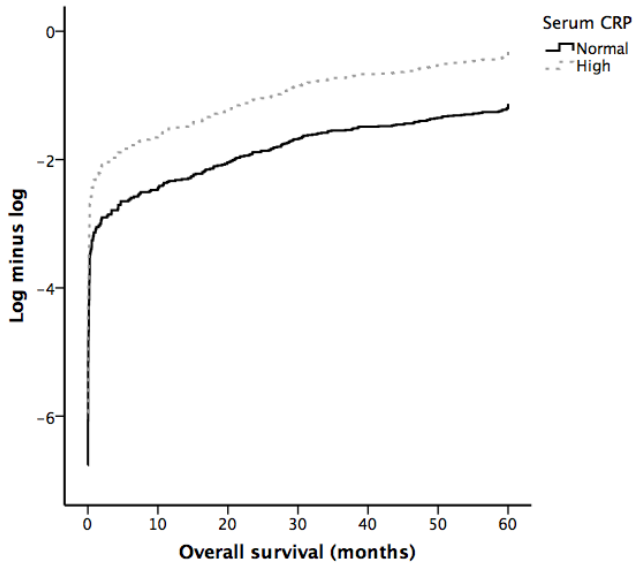


Figure 3.25: Log minus log plot of serum CRP and overall survival

Log(-log(survival)) plot demonstrating near proportionality of survival between the two groups

Despite the significant associations observed between serum CRP and poor survival, its predictive value is relatively poor. When ROC analysis was performed using death as the endpoint, the AUC was 0.63 (95% CI 0.58 – 0.68, p<0.001) for serum CRP as a categorical variable and an AUC of 0.61 (95% CI 0.56 – 0.66, p<0.001) for CRP as a continuous variable (figure 3.26).

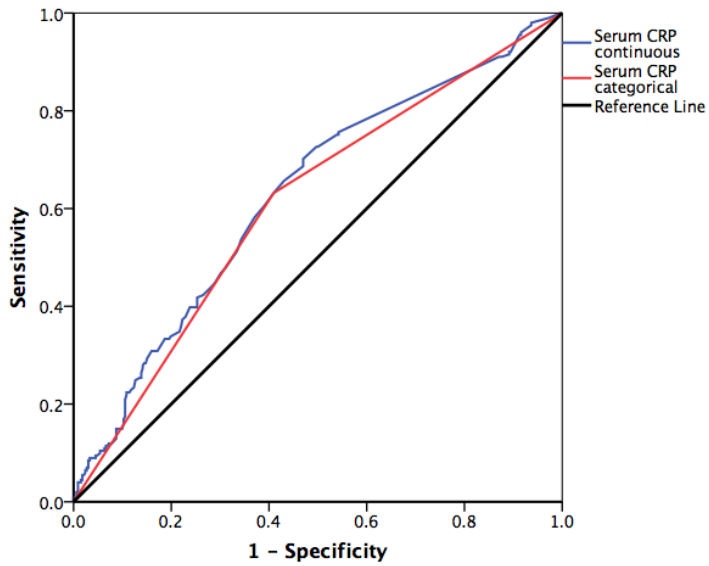


Figure 3.26: Predictive value of CRP in identifying patients who will die during follow-up

Receiver-operator-characteristic curve demonstrating the predictive value of serum CRP in identifying patients who will die during follow-up.

3.4.4.5 Univariable overall survival - serum albumin expression

Serum albumin measurements were significantly lower in patients who died during the follow up period (p<0.001, Mann-Whitney U, figure 3.27). Median serum albumin measurements were 40 g/l (IQR 33 – 47 g/l) in the alive group compared to 37 g/l (IQR 27 – 47 g/l) in patients who died during follow-up.

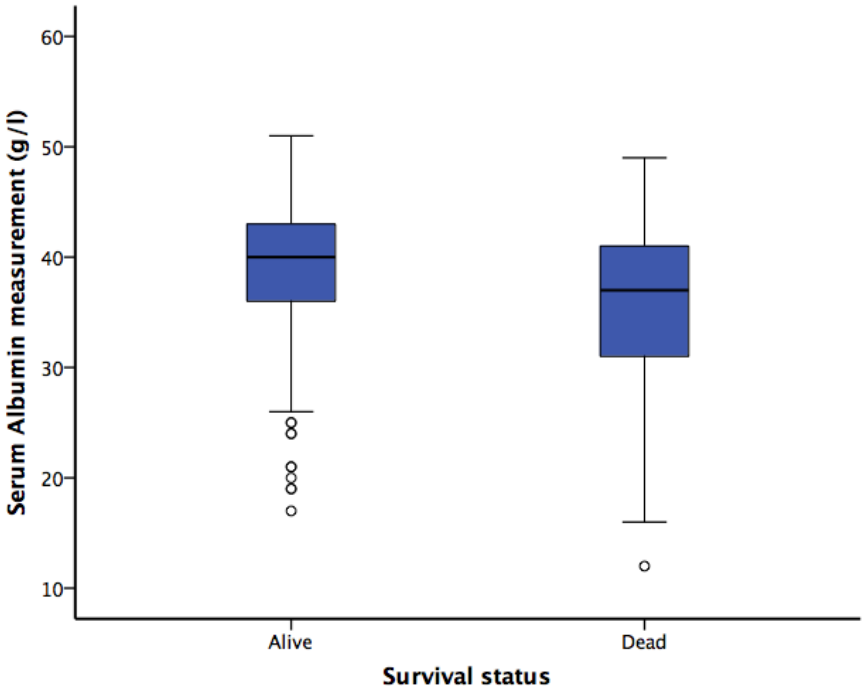
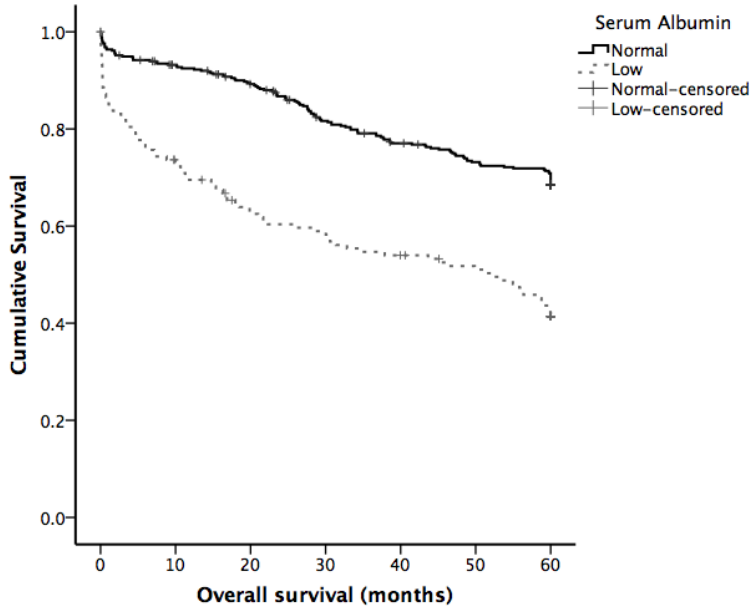


Figure 3.27: The distribution of serum albumin measurements in patients stratified by survival status (p<0.001)

The 5 year overall survival rate for patients with hypoalbuminaemia was 38.0% compared to 66.1% (p<0.001, Pearson's chi square) in patients with a normal serum albumin. On logrank analysis hypoalbuminaemia was significantly associated with poorer overall survival (p<0.001, figure 3.28). The mean survival for patients with hypoalbuminaemia was 36.3 months (95% CI 32.2 – 45.5) compared with 49.9 months (95% CI 48.1 – 51.7) in the normal serum albumin group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	413	378	357	320	300	283	274
Low	150	106	89	83	75	69	57

Figure 3.28: The relationship between serum albumin expression and overall survival in patients with colorectal cancer (p<0.001)

Kaplan-Meier curve demonstrating the proportion of patients surviving stratified by serum albumin expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis hypoalbuminaemia was significantly associated with poor overall survival when dichotomised as a categorical variable (HR 2.50 (95% CI 1.90 – 3.30), p<0.001). Furthermore, it was also significantly associated with poor overall survival when included as a continuous variable (HR 0.94 (95% CI 0.92 – 0.95), p<0.001). The results of the Kaplan-Meier plot suggest that serum albumin violates the proportionality assumption of the Cox model. There appears to be a greater proportion of events in the hypoalbuminaemic group near the origin of the curve. The subsequent gradient associated with these events does not hold throughout the follow up period suggesting that the hazard ratio is not constant thus violating proportionality. When assessing the log(-log(survival)) plot (figure 3.29), categorical serum albumin demonstrates parallel curves, however, there is evidence of time dependency when an interaction term between serum albumin and log(time) was placed in a Cox proportional hazard model (HR 0.98 (95% CI 0.97 – 1.00); p=0.032) with an associated adjustment of the HR for categorical serum albumin (HR 3.62 (95% CI 2.35 – 5.58); p<0.001) (table 3.7).

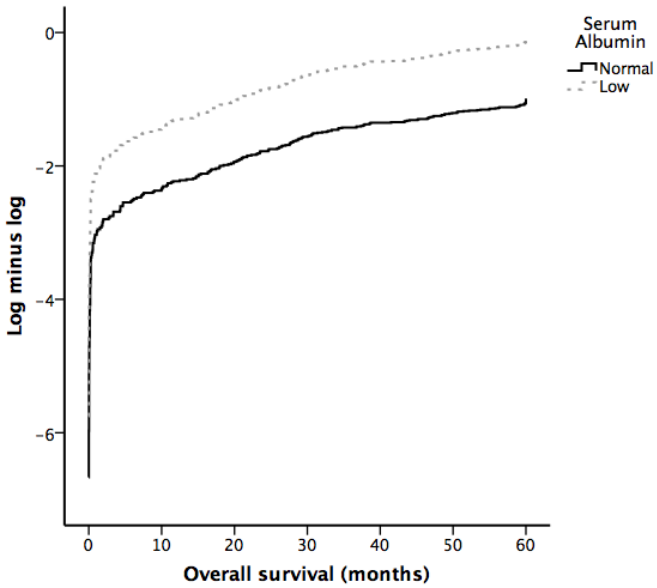


Figure 3.29: Log minus log plot of serum albumin and overall survival

Log(-log(survival)) plot demonstrating near proportionality of survival between the two groups.

In terms of survival prediction, when ROC analysis was performed using death as the endpoint, the AUC was 0.35 (95% CI 0.31 – 0.40, p<0.001) for albumin as a continuous variable and an AUC of 0.61 (95% CI 0.56 – 0.66, p<0.001) for albumin as a categorical variable (figure 3.). 30

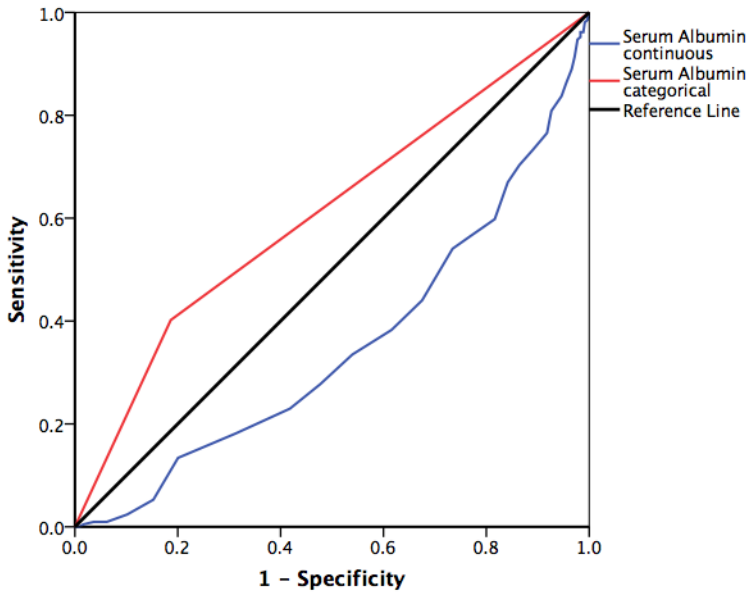
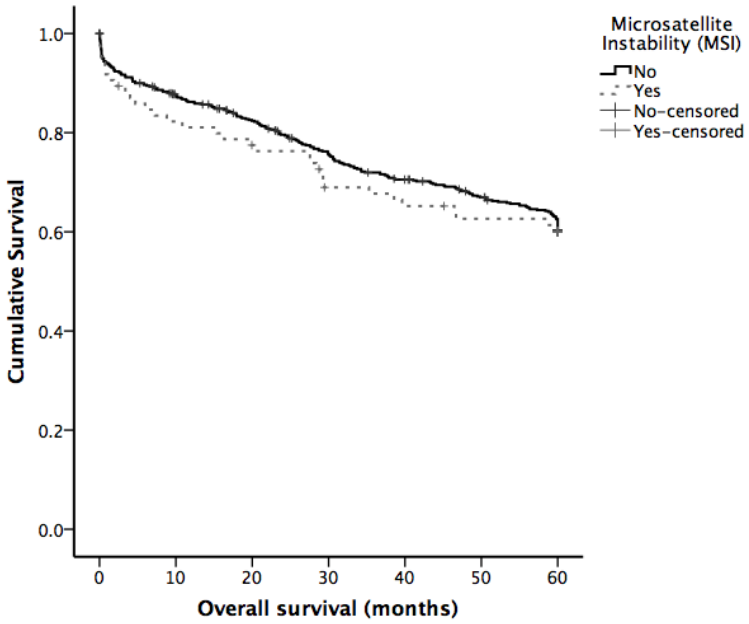


Figure 3.30: Predictive value of serum albumin in identifying patients who will die during follow-up

Receiver-operator-characteristic curve demonstrating the predictive value of serum albumin in identifying patients who will die during follow-up.

3.4.4.6 Univariable overall survival - tumour MSI status

Of the patients with MSI tumours, 33 (38.8%) died during the follow up period compared with 227 (38.3%) of the patients with CI tumours (p=0.932, Fisher's exact test). The 5 year survival rate for patients with MSI tumours was 55.3% compared to 57.8% (p=0.666, Pearson's chi square) of patients with CI tumours. On logrank analysis, MSI status was not associated with overall survival (p=0.760, figure 3.31). The mean survival for patients with MSI tumours was 43.7 months (95% CI 38.8 – 48.7) compared with 46.2 months (95% CI 44.4 – 47.9) in the CI group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
CI	592	508	471	427	395	370	343
MSI	85	69	64	55	52	49	47

Figure 3.31: The relationship between tumour MSI status and overall survival

Kaplan-Meier curve demonstrating the proportion of patients surviving stratified by tumour MSI status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis MSI status was not significantly associated with improved overall survival (HR 1.03 (95% CI 0.86 – 1.24), p=0.760). Furthermore, MSI status did not significantly predict death during follow-up with an AUC of 0.50 (95% CI 0.46 – 0.55, p=0.961).

3.4.4.7 Multivariable recurrence free survival

Proportionality assumptions testing

All the studied variables underwent Cox proportional hazards assumption testing as described in the statistical methodology section 2.7.8.7.

Cox proportional hazards regression analysis

All covariates with a significance level of $p<0.1$ were included in the multivariable model. Terms were removed if their respective p-value was >0.05. On univariable analysis, emergency presentation ($p<0.001$), advancing T-stage ($p<0.001$), advancing N-stage ($p<0.001$), higher TNM stage ($p<0.001$), poor differentiation ($p=0.007$), raised serum CRP ($p=0.013$), hypoalbuminaemia ($p=0.011$), poor Klintrup score ($p<0.001$) and tumour MSI ($p=0.035$) had a p-value <0.1 and were therefore included in the multivariable Cox proportional hazards regression model.

On multivariable analysis, only advancing T-stage (when all subcategories were compared with T1 (T2 HR 0.59 (95% CI 0.11 – 3.04), (T3 HR 0.97 (95% CI 0.23 – 4.09) and (T4 HR 2.69 (95% CI 0.64 – 11.34)), $p<0.001$), N-stage (when all subcategories were compared with N0 (N1 HR 2.40 (95% CI 1.58 – 4.09), (N2 HR 3.45 (95% CI 2.08 – 5.73), $p<0.001$) and Klintrup score (HR 0.34 (95% CI (0.32 – 0.84);$p=0.008$) were independently and significantly associated with poor overall survival (table 3.7).

Table 3.7: The relationship between clinicopathological factors and recurrence-free survival in patients with colorectal cancer: univariable and multivariable analysis

	Univariable analysis			Multivariable analysis		
	Coefficient	p-value	Hazard ratio (95% CI)	Coefficient	p-value	Hazard ratio (95% CI)
Age	-0.120	0.101	0.99 (0.98 - 1.00)			
Sex						
Female			1			
Male	0.121	0.461	1.13 (0.82 – 1.56)			
Presentation						
Elective			1			
Emergency	0.689	<0.001	1.99 (1.477 – 2.0)		0.224	
Tumour site						
Right	0.000	0.278	1			
Left	-0.249		0.78 (0.53 – 1.14)			
Rectum	0.085		1.09 (0.73 – 1.62)			
T stage						
1	0.000	<0.001	1	0.000	<0.001	1
2	-0.008		0.99 (0.21 – 4.77)	-0.532		0.59 (0.11 – 3.04)
3	1.010		2.75 (0.67 – 11.20)	-0.028		0.97 (0.23 – 4.09)
4	1.944		6.99 (1.71 – 28.49)	0.990		2.69 (0.64 – 11.34)
N stage						
0	0.000	<0.001	1	0.000	<0.001	1
1	1.133		3.12 (2.16 – 4.47)	0.874		2.40 (1.58 – 3.63)
2	1.702		5.49 (3.59 – 8.39)	1.239		3.45 (2.08 – 5.73)
TNM stage						
I	0.000	<0.001	1			
II	0.953		2.60 (1.18 – 5.70)			
III	2.051		7.78 (3.60 – 16.80)		0.801	
Differentiation						
Well/Moderate			1			
Poor	0.629	0.007	1.88 (1.18 – 2.98)		0.853	
Serum CRP						
Normal			1			
High	0.458	0.013	1.58 (1.10 – 2.27)		0.964	
Serum albumin						
Normal			1			
Low	0.495	0.011	1.64 (1.12 – 2.40)		0.064	
Klintrup score						
High			1			1
Low	-1.091	<0.001	0.34 (0.22 – 0.52)	-0.664	0.008	0.52 (0.32 – 0.84)
MSI status						
CI			1			
MSI	-0.689	0.035	0.50 (0.26 – 0.95)		0.377	

3.4.4.8 Multivariable overall survival

Proportionality assumptions testing

All the studied variables underwent Cox proportional hazards assumption testing as described in the statistical methodology section 2.7.8.7. Presentation, serum CRP and Serum albumin demonstrated time dependency when interaction terms between the covariates and log(time) were placed in the model.

Cox proportional hazards regression analysis

All covariates with a significance level of $p<0.1$ were included in the multivariable model. Terms were removed if their respective p-value was >0.05. On univariable analysis, advancing age ($p<0.001$), emergency presentation ($p<0.001$), advancing T-stage ($p<0.001$), advancing N-stage ($p=0.001$), higher TNM stage ($p<0.001$), poor differentiation ($p=0.001$), raised serum CRP ($p<0.001$), hypoalbuminaemia ($p<0.001$) and good Klintrup score ($p<0.001$) had a p-value <0.1 and were therefore included in the multivariable Cox proportional hazards regression model.

On multivariable analysis, emergency presentation (HR 2.36 (95% CI 1.49 – 3.72), $p<0.001$), hypoalbuminaemia (HR 1.65 (95% CI 1.20 – 2.26), $p=0.002$), Klintrup score (HR 0.59 (95% CI 0.41 – 0.85), $p=0.004$), advancing T-stage (when all subcategories were compared with T1 (T2 HR 1.09 (95% CI 0.31 – 3.80), (T3 HR 1.25 (95% CI 0.39 – 4.01) and (T4 HR 2.00 (95% CI 0.61 – 6.52)), $p=0.015$) and N-stage (when all subcategories were compared with N0 (N1 HR 1.31 (95% CI 0.95 – 1.83), (N2 HR 1.86 (95% CI 1.23 – 2.79), $p=0.010$) were independently and significantly associated with poor overall survival (table 3.8).

Influence of time dependency on outcome

Only emergency presentation ($p=0.014$) retained significant time dependency when placed in the multivariable analysis with serum CRP ($p=0.567$) and serum albumin ($p=0.190$) becoming non-significant.

Table 3.8: The relationship between clinicopathological factors and overall survival in patients with colorectal cancer: univariable and multivariable analysis

	Univariable analysis			Multivariable analysis		
	Coefficient	p-value	Hazard ratio (95% CI)	Coefficient	p-value	Hazard ratio (95% CI)
Age	0.035	<0.001	1.04 (1.02 - 1.05)	0.039	<0.001	1.04 (1.03 – 1.06)
Sex						
Female			1			
Male	-0.036	0.774	0.97 (0.76 – 1.23)			
Presentation						
Elective			1			1
Emergency	1.336	<0.001	3.80 (2.58 – 5.62)	0.858	<0.001	2.36 (1.49 – 3.72)
Presentation x log(time)	-0.022	0.001	0.98 (0.97 – 0.99)	-0.187	0.014	0.83 (0.72 – 0.96)
Tumour site						
Right	(0.000)	0.509	1			
Left	-0.161		0.85 (0.64 – 1.13)			
Rectum	-0.109		0.90 (0.65 – 1.23)			
T stage						
1	(0.000)	<0.001	1	(0.000)	0.015	1
2	-0.111		0.90 (0.38 – 2.09)	0.089		1.09 (0.31 – 3.80)
3	0.355		1.43 (0.67 – 3.05)	0.222		1.25 (0.39 – 4.01)
4	0.292		2.64 (1.23 – 5.67)	0.691		2.00 (0.61 – 6.52)
N stage						
0	(0.000)	<0.001	1	(0.000)	0.010	1
1	0.492		1.64 (1.24 – 2.16)	0.273		1.31 (0.95 – 1.83)
2	0.954		2.60 (1.84 – 3.66)	0.618		1.86 (1.23 – 2.79)
TNM stage						
I	(0.000)	<0.001	1		0.901	
II	0.437		1.55 (1.00 – 2.39)			
III	0.362		2.63 (1.71 – 4.05)			
Differentiation						
Well/Moderate			1		0.209	
Poor	0.598	0.001	1.82 (1.28 – 2.59)			
Serum CRP						
Normal			1			1
High	1.440	<0.001	4.22 (2.56 – 6.95)	0.305	0.076	1.36 (0.97 – 1.90)
CRP x log(time)	-0.025	0.002	0.98 (0.96 – 0.99)		0.567	
Serum albumin						
Normal			1			1
Low	1.286	<0.001	3.62 (2.35 – 5.58)	0.498	0.002	1.65 (1.20– 2.26)
albumin x log(time)	-0.016	0.032	0.98 (0.97 – 1.00)		0.190	
Klintrup score						
High			1			1
Low	-0.749	<0.001	0.47 (0.35 – 0.64)	-0.531	0.004	0.59 (0.41 – 0.85)
MSI status						
CI			1			
MSI	0.057	0.760	1.06 (0.74 – 1.53)			

3.4.5 The associations of serum CRP and albumin, MSI status, clinicopathological factors and survival stratified by TNM stage

Lymph node metastasis is a key determinant that influences the decision to offer adjuvant chemotherapy therapy to patients who have undergone potentially curative surgery. Such patients are largely AJCC TNM stage I, II or III. A particular clinical challenge is establishing a robust treatment plan for patients with stage II disease, as their outcome is heterogeneous. Adjuvant chemotherapy may cause adverse events and therefore offering this treatment to all patients with stage II disease is not considered appropriate.

A statistical model of survival prediction better than the currently used TNM staging system, or which offers a useful adjunct to the TNM subgroups is particularly needed. Therefore, the associations and survival outcomes of the biomarkers discussed within this chapter were analysed according to TNM stage. The relatively few patients with stage I colorectal cancer within this cohort have very few events, increasing the risk of type I and type II errors. Therefore, survival and association measures were only analysed for patients with TNM stage II and III cancers. Of the 677 patients studied in cohort 2 (validation cohort) 327 (48.3%) were TNM stage II and 238 (35.2%) were TNM stage III.

3.4.5.1 Serum CRP expression stratified by TNM stage II and III

There was no significant difference in serum CRP between patients with TNM stage II and III colorectal cancer (p=0.610, Mann-Whitney U, supplementary figure 3.12, appendix 5.1). The serum CRP measurements in patients with stage II colorectal cancer ranged from 0.90 – 269.00 mg/l with median of 12.00 mg/l (IQR 0.90 – 30.25 mg/l) compared with a range of 1.00 – 460.00 mg/l and median of 11.00 mg/l (IQR 1.00 – 42.50 mg/l) in patients with stage III colorectal cancer. This was also observed by chi-squared analysis when serum CRP was analysed as a categorical variable (p=0.558, table 3.9).

Table 3.9: The relationship between TNM stage and clinicopathological factors

Clinicopathological factors	TNM stage II	TNM stage III	p-value
Age	72 (IQR 57 – 87)	73 (IQR 56 – 90)	0.388
Sex			
Female	158 (48.3%)	124 (50.6%)	0.587
Male	169 (51.7%)	121 (49.4%)	
Presentation			
Elective	221 (67.6%)	158 (64.5%)	0.439
Emergency	106 (32.4%)	87 (35.5%)	
Tumour Site			
Right	147 (45.0%)	121 (49.4%)	0.564
Left	117 (35.8%)	82 (33.5%)	
Rectum	63 (19.3%)	42 (17.1%)	
Differentiation			
Well-Mod	299 (91.4%)	208 (84.9%)	0.015
Poor	28 (8.6%)	37 (15.1%)	
T stage			
1	0 (0.0%)	1 (0.4%)	<0.001
2	0 (0.0%)	12 (4.9%)	
3	225 (68.8%)	136 (55.5%)	
4	102 (31.2%)	96 (39.2%)	
Serum CRP			
Normal	122 (46.7%)	96 (49.7%)	0.527
High	139 (53.3%)	97 (50.3%)	
Serum albumin			
Normal	194 (71.3%)	148 (72.2%)	0.834
Low	78 (28.7%)	57 (27.8%)	
Klintrup score			
High	233 (71.3%)	194 (79.2%)	0.031
Low	94 (28.7%)	51 (20.8%)	
MSI Status			
CI	277 (84.7%)	224 (91.4%)	0.016
MSI	50 (15.3%)	21 (8.6%)	

Bonferroni adjustment = <0.005
* Fishers exact test

3.4.5.2 Serum albumin expression stratified by TNM stage II and III colorectal cancer

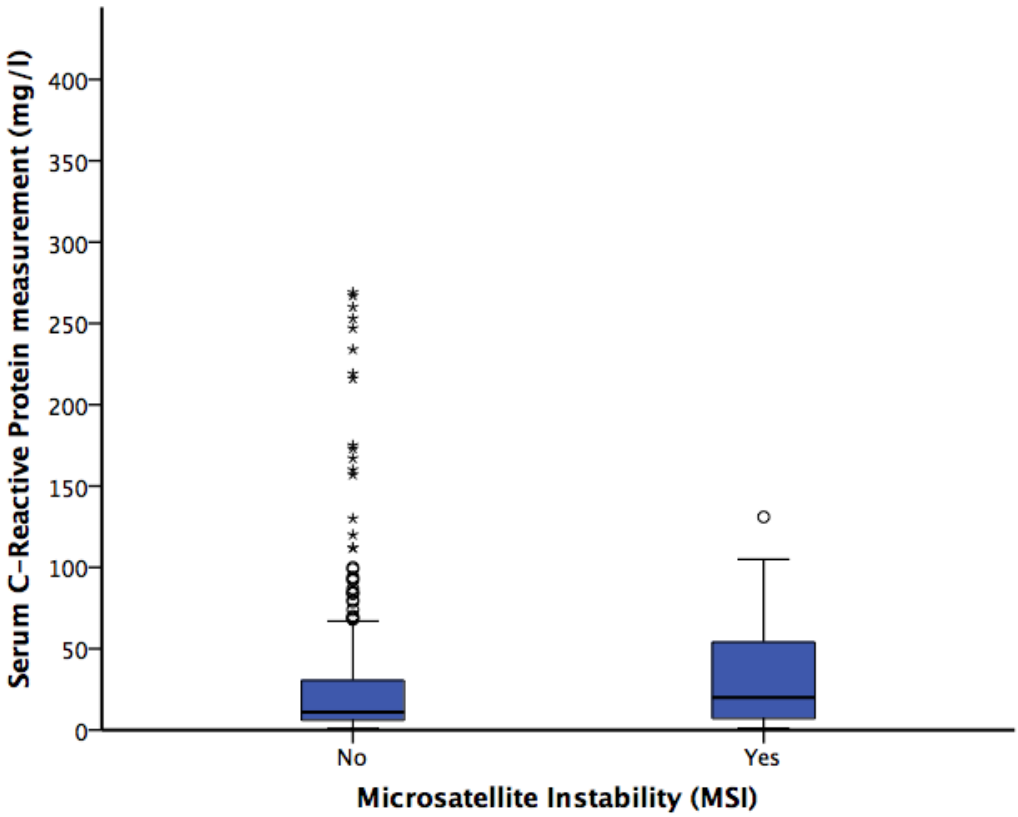
Serum albumin measurements were not significantly lower in the stage III group compared with the stage II group (p=0.393, Mann-Whitney U, supplementary figure 3.13, appendix 5.1). Measurements of serum albumin ranged from 18 – 51 g/l with a median of 39 g/l (IQR 30 – 48 g/l) in TNM stage II compared with a range of 12 – 51 g/l and a median of 38 g/l (IQR 29 – 47 g/l) in patients with TNM stage III. This was also observed by chi-squared analysis when serum albumin was analysed as a categorical variable (p=0.552, table 3.8).

3.4.5.3 MSI and clinicopathological factor proportionality stratified by TNM stage II and III

Following Bonferroni adjustment of α to 0.005 none of the clinicopathological variables studied demonstrated a significant relationship to TNM stage (table 3.9).

3.4.5.4 The association of MSI status and clinicopathological factors stratified in TNM stage II colorectal cancer

Patients with MSI tumours did not have significantly higher levels of serum CRP (median of 20.00 mg/l (IQR 1.00 – 70.00 mg/l)) when compared with CI tumours (median of 11.00 mg/l (IQR 0.90 – 35.75 mg/l)) (p=0.099, Mann-Whitney U, figure 3.32). This trend (but not significant association) was also observed by chi-squared analysis when the variables were analysed as categorical data (p=0.036, Bonferroni adjustment p≤0.005). On chi-squared analysis of categorical variables, right-sided tumour location (p<0.001) and poor differentiation (p<0.001) were significantly associated with MSI tumours following Bonferroni adjustment (table 3.10). When serum albumin was analysed as a continuous variable, there was no statistically significant difference between serum measurements and MSI status (p=0.295, Mann-Whitney U, figure 3.32).



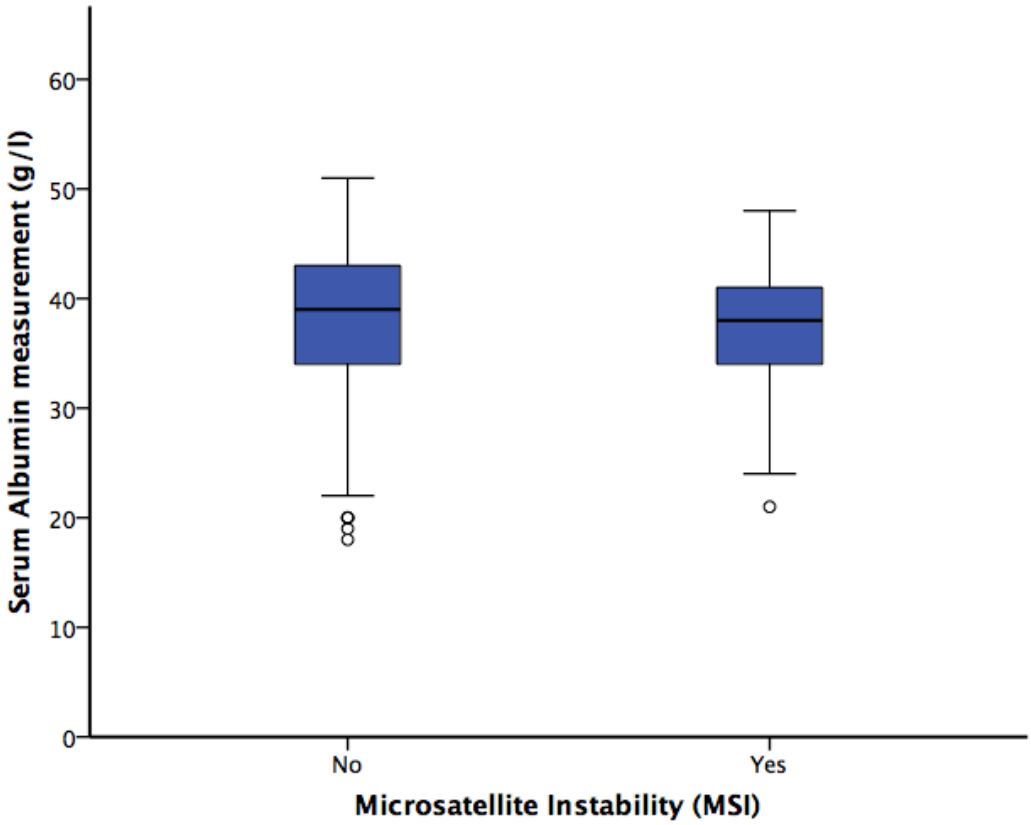


Figure 3.32: The distribution of serum CRP and albumin stratified by MSI status in patients with TNM stage II colorectal cancer (p=0.099)

Table 3.10: The relationship between tumour MSI status and categorical clinicopathological factors in patients with stage II colorectal cancer

Clinicopathological factors	Chromosomal Instability (CI)	Microsatellite Instability (MSI)	p-value
Age	73 (IQR 58 – 88)	71 (IQR 55 – 87)	0.612
Sex			
Female	125 (45.1%)	33 (66.0%)	0.007
Male	152 (54.9%)	17 (34.0%)	
Presentation			
Elective	183 (66.1%)	38 (76.0%)	0.167
Emergency	94 (33.9%)	12 (24.0%)	
Tumour Site			
Right	103 (37.2%)	44 (88.0%)	**<0.001**
Left	114 (41.2%)	3 (6.0%)	
Rectum	60 (21.7%)	3 (6.0%)	
Differentiation			
Well-Mod	261 (94.2%)	38 (76.0%)	<0.001
Poor	16 (5.8%)	12 (24.0%)	
T stage			
1	N/A	N/A	0.144
2	N/A	N/A	
3	195 (70.4%)	30 (60.0%)	
4	82 (29.6%)	20 (40.0%)	
Serum CRP			
Normal	109 (49.5%)	13 (31.7%)	0.036
High	111 (50.5%)	28 (68.3%)	
Serum albumin			
Normal	166 (71.9%)	28 (68.3%)	0.641
Low	65 (28.1%)	13 (31.7%)	
Klintrup score			
High	199 (71.8%)	34 (68.0%)	0.581
Low	78 (28.2%)	52 (32.0%)	

Bonferoni adjustment = ≤0.006

* Fishers exact test

3.4.5.5 The association of MSI status and clinicopathological factors in patients with TNM stage III colorectal cancer

Patients with MSI tumours had significantly higher levels of serum CRP (median 35.00 mg/l (IQR 6.00 – 79.50 mg/l)) when compared with CI tumours (median 9.50 mg/l (IQR 1.00 – 36.75 mg/l)) (p=0.002, Mann-Whitney U, figure 3.33). This association was also supported by chi-squared analysis when the variables were analysed as categorical data (p=0.006, Bonferroni adjustment p≤0.006, table 3.11). Furthermore, serum albumin measurements were lower in patients with MSI tumours (median 34 g/l (IQR 22 – 46 g/l)) when compared with CI tumours (median 39 g/l (IQR 31 – 47 g/l)) (p=0.036, Mann-Whitney U, figure 3.33). This association was also supported by chi-squared analysis when

the variables were analysed as categorical data (p=0.003, Bonferroni adjustment p≤0.006, table 3.10). On chi-squared analysis of categorical variables, none of the frequencies of the clinicopathological factors studied were significantly different between patients with MSI or CI colorectal cancer (table 3.10).

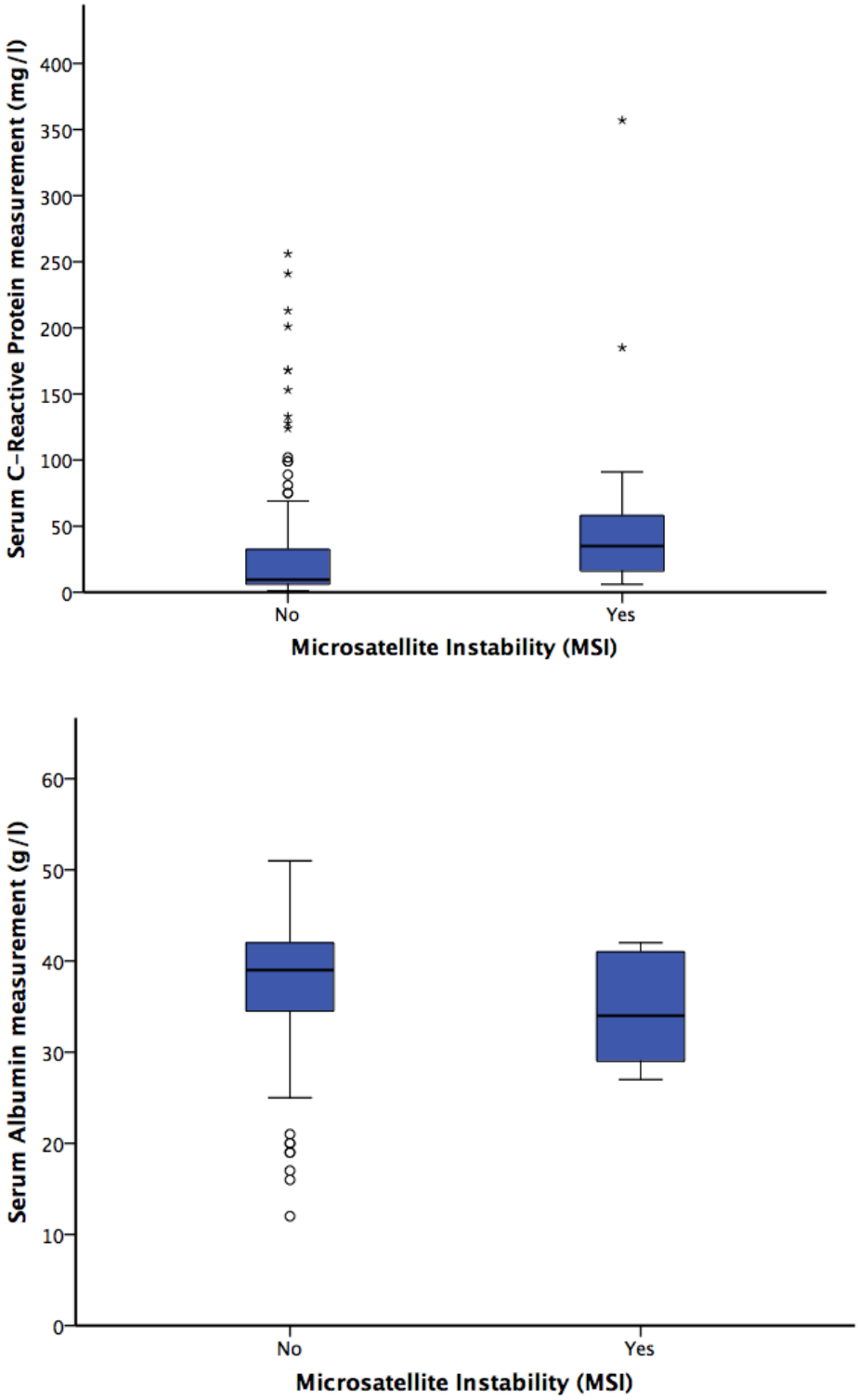


Figure 3.33: The distribution of serum CRP and albumin stratified by MSI status in patients with TNM stage III colorectal cancer

Table 3.11: The relationships between tumour MSI status and categorical clinicopathological factors in patients with stage III colorectal cancer

Clinicopathological factors	Chromosomal Instability (CI)	Microsatellite Instability (MSI)	p-value
Age	73 (IQR 55 – 91)	75 (IQR 62 – 88)	0.358
Sex			
Female	111 (49.6%)	13 (61.9%)	0.279
Male	113 (50.4%)	8 (38.1%)	
Presentation			
Elective	146 (65.2%)	12 (57.1%)	0.462
Emergency	78 (34.8%)	9 (42.9%)	
Tumour Site			
Right	106 (47.3%)	15 (71.4%)	0.107
Left	78 (34.8%)	4 (19.0%)	
Rectum	40 (17.9%)	2 (9.5%)	
Differentiation			
Well-Mod	193 (86.2%)	15 (71.4%)	0.071
Poor	31 (13.8%)	6 (28.6%)	
T stage			
1	1 (0.4%)	0 (0%)	0.857
2	11 (4.9%)	1 (4.8%)	
3	126 (56.2%)	10 (47.6%)	
4	86 (38.4%)	10 (47.6%)	
Serum CRP			
Normal	93 (52.8%)	3 (17.6%)	0.006
High	83 (47.2%)	14 (82.4%)	
Serum albumin			
Normal	141 (75.0%)	7 (41.2%)	0.003
Low	47 (25.0%)	10 (58.8%)	
Klintrup score			
High	182 (81.2%)	12 (57.1%)	0.009
Low	42 (18.8%)	9 (42.9%)	

Bonferoni adjustment = ≤0.006

* Fishers exact test

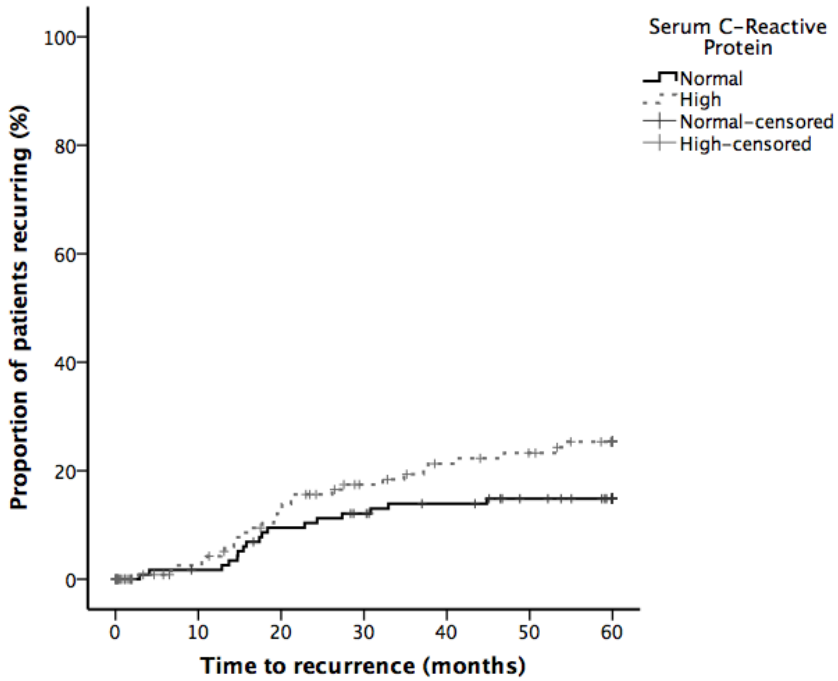
3.4.5.6 The association of serum CRP, albumin, MSI status and survival stratified by TNM stage II

During follow-up there were 54 (16.5%) recurrences and 114 (34.9%) deaths. Five year recurrence-free and overall survival rates for the clinicopathological factors studied can be found in appendix 3.3. Based on a 15% difference in 5 year survival with an α=0.05 and β=0.2 the number of patient required was n=338. For a multivariable model containing at least 6 variables a minimum of 60 events are required. Based on a sample size of n=327 at least 75% power is available.

Univariable recurrence-free survival - serum CRP expression

Serum CRP measurements were not significantly different in patients who developed disease recurrence (p=0.230, Mann-Whitney U, supplementary figure 3.14, appendix 5.1). The median for patients with recurrence was 15.00 mg/l (IQR 2.00 – 66.50 mg/l) compared with 11.00 mg/l (IQR 0.90 – 36.75 mg/l) in the non-recurrence group.

The 5 year recurrence-free survival rate for patients with a raised serum CRP was 49.6% compared to 67.2% in patients with a normal serum CRP (p=0.004, Pearson's chi square). On logrank analysis raised serum CRP was not significantly associated with poor recurrence-free survival (p=0.065, figure 3.34). The mean survival for patients with a raised serum CRP was 50.9 months (95% CI 47.8 – 64.1) compared with 54.0 months (95% CI 51.3 – 56.7) in the normal serum CRP group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	122	114	104	99	94	88	82
High	139	114	100	87	80	76	69

Figure 3.34: The relationship between serum CRP expression and recurrence-free survival in patients with stage II colorectal cancer

Kaplan-Meier curve demonstrating the proportion of patients recurring stratified by serum CRP expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

Similar to logrank analysis Cox univariable regression analysis demonstrated that a raised serum CRP was not significantly associated with poor recurrence-free survival (HR 1.75 (95% CI 0.96 – 3.20), p=0.068) when dichotomised as a categorical variable. The hazard

ratio became statistically significant when it was included as a continuous variable (HR 1.01 (95% CI 1.00 – 1.01), p=0.031). Despite the significant associations observed between serum CRP and disease recurrence, its predictive value remains poor. When ROC analysis was performed using recurrence as the endpoint, the AUC was 0.56 (95% CI 0.46 – 0.65, p=0.233) for CRP as a continuous variable and the AUC was 0.55 (95% CI 0.46 – 0.65, p=0.253) for CRP as a categorical variable (supplementary figure 3.15, appendix 5.1).

Univariable recurrence-free survival - serum albumin

There was no statistical significance between the serum albumin measurements of patients who did and did not develop cancer recurrence during follow-up (p=0.236, Mann-Whitney U, supplementary figure 3.16, appendix 5.1). Median serum albumin was 39 g/l (IQR 30 – 48 g/l) in the non-recurrence group compared to 38 g/l (IQR 31 – 46 g/l) in the recurrence group.

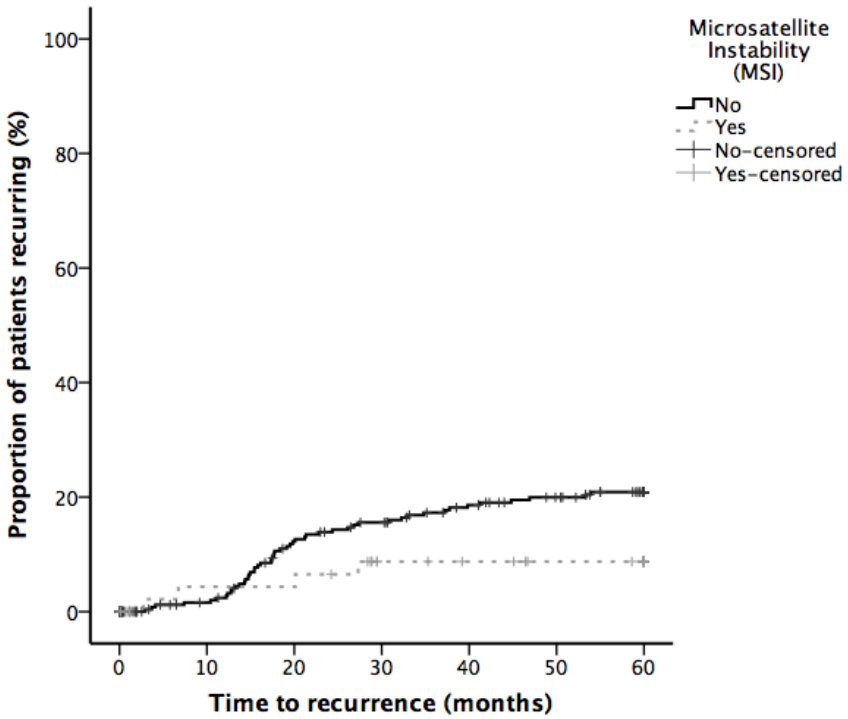
The 5 year recurrence-free survival rate for patients with hypoalbuminaemia was 49.6% compared to 67.2% in patients with a normal serum albumin (p=0.004, Pearson's chi square). On logrank analysis hypoalbuminaemia was not associated with recurrence-free survival (p=0.349). The mean survival for patients with hypoalbuminaemia was 52.1 months (95% CI 48.1 – 56.1) compared with 52.6 months (95% CI 50.2 – 54.9) in the normal serum albumin group (supplementary figure 3.17, appendix 5.1).

On Cox univariable regression analysis hypoalbuminaemia was not significantly associated with recurrence-free survival when dichotomised as a categorical variable (HR 1.35 (95% CI 0.72 – 2.52), p=0.350). The hazard ratio was statistically significant when analysed as a continuous variable (HR 0.96 (95% CI 0.92 – 1.00), p=0.038). There was no statistically significant predictive association between serum albumin and disease recurrence when analysed using ROC analysis. With disease recurrence as the endpoint, the AUC was 0.51 (95% CI 0.42 – 0.60, p=0.885) for albumin as a categorical variable and an AUC of 0.45 (95% CI 0.36 – 0.53, p=0.237) for albumin as a continuous variable (supplementary figure 3.18, appendix 5.1).

Univariable recurrence-free survival - Tumour MSI status

Of the patients with MSI tumours, 4 (8.0%) developed cancer recurrence compared with 50 (18.1%) of the patients with CI tumours (p=0.053, Fisher's exact test). The 5 year recurrence-free survival rate for patients with MSI tumours was 60.0% compared to 58.1% in patients with CI tumours (p=0.805, Pearson's chi square). On logrank analysis, MSI

status was not significantly associated with improved recurrence-free survival (p=0.082, figure 3.35). The mean survival for patients with MSI tumours was 56.0 months (95% CI 52.2 – 59.8) compared with 52.1 months (95% CI 50.1 – 54.2) in the CI group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
CI	277	243	212	200	185	175	161
MSI	50	44	44	36	34	31	30

Figure 3.35: The relationship between MSI status and recurrence-free survival in patients with stage II colorectal cancer (p=0.082)

Kaplan-Meier curve demonstrating the proportion of patients recurring stratified by MMR protein status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis, MSI tumours were not significantly associated with improved recurrence free survival (HR 0.42 (95% CI 0.15 – 1.15), p=0.092); furthermore, MSI status did not significantly predict the development of recurrence with an AUC of 0.45 (95% CI 0.37 – 0.53, p=0.273) (supplementary figure 3.18, appendix 5.1).

Univariable overall survival - serum CRP expression

Serum CRP measurements were significantly higher in patients who died during follow-up (p=0.015, Mann-Whitney U, figure 3.36). The median for patients who died was 16.00 mg/l (IQR 1.00 – 69.75 mg/l) compared with 9.00 mg/l (IQR 0.90 – 30.00 mg/l) in the alive group.

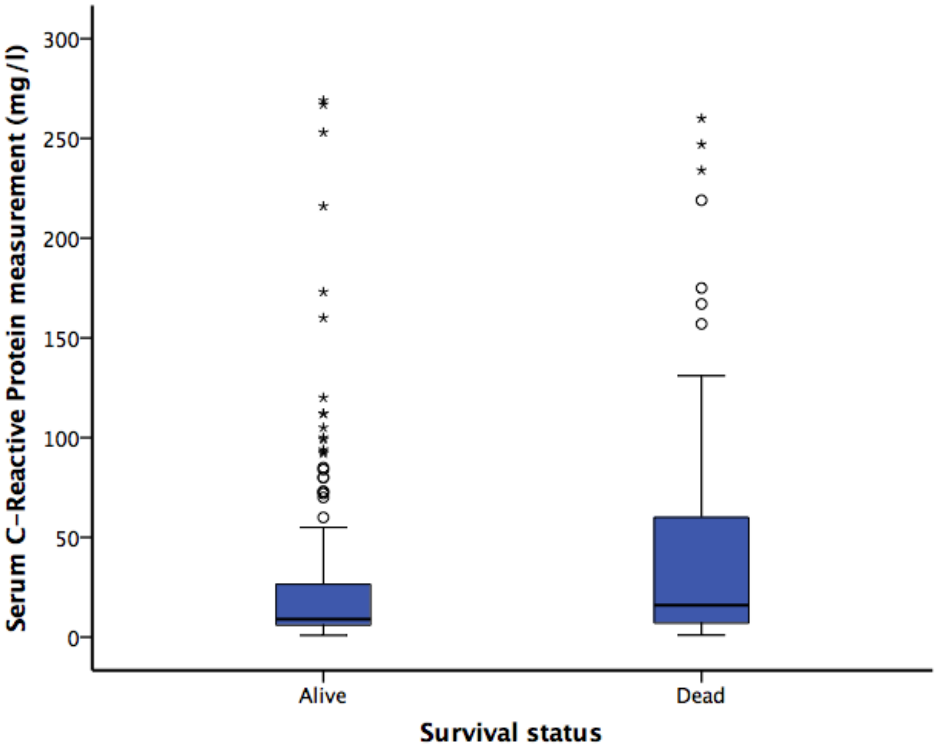
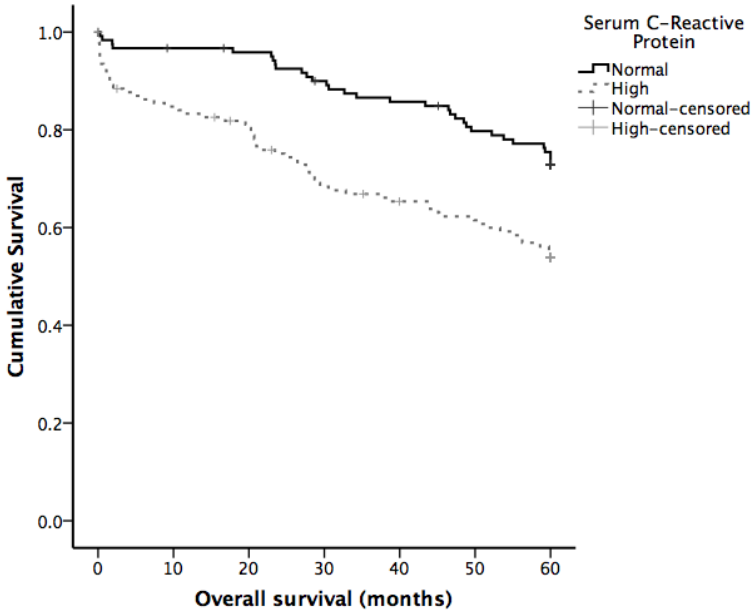


Figure 3.36: The distribution of serum CRP measurements in patients stratified by survival status (p=0.015)

The 5 year overall survival rate for patients with a raised serum CRP was 51.1% compared to 72.1% in patients with a normal serum CRP (p=0.001, Pearson's chi square). On logrank analysis raised serum CRP was significantly associated with poor overall survival (p<0.001, figure 3.37). The mean survival for patients with a raised serum CRP was 39.6 months (95% CI 36.7 – 42.6) compared with 51.9 months (95% CI 49.9 – 54.0) in the normal serum CRP group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	122	116	114	106	101	93	88
High	139	116	108	91	85	80	71

Figure 3.37: The relationship between serum CRP expression and overall survival in patients with stage II colorectal cancer (p<0.001)

Kaplan-Meier curve demonstrating the proportion of patients surviving stratified by serum CRP expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis raised serum CRP was significantly associated with poor overall survival when dichotomised as a categorical variable (HR 2.11 (95% CI 1.38 – 3.23), p=0.001). The hazard ratio was less significant when it was included as a continuous variable (HR 1.00 95% CI 1.00 – 1.01, p=0.020). The results of the Kaplan-Meier plot, however, suggest that serum CRP violates the proportionality assumption of the Cox proportional hazards model. There appears to be a greater proportion of events in the high group near the origin of the curve. The subsequent gradient associated with these events does not hold throughout the follow up period suggesting that the HR is not constant and thus violating proportionality. When assessing the log(-log(survival)) plot (figure 3.38), categorical serum CRP demonstrates parallel curves, however, however, there is evidence of time dependency when an interaction term between serum CRP and log(time) was placed in a Cox proportional hazard model (HR 0.73 (95% CI 0.54 – 1.00); p=0.051) with an associated adjustment of the HR for categorical serum CRP (HR 5.04 (95% CI 1.78 – 14.24); p=0.002).

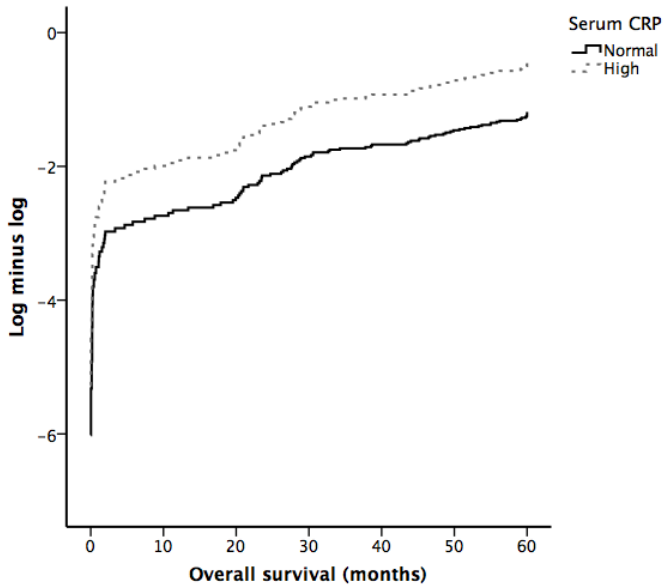


Figure 3.38: Log minus log plot of serum CRP and overall survival in patients with stage II colorectal cancer

Log(-log(survival)) plot demonstrating near proportionality of survival between the two groups

Despite the significant associations observed between serum CRP and poor survival, its predictive value is relatively poor. When ROC analysis was performed using death as the endpoint, the AUC was 0.60 (95% CI 0.53 – 0.67, p=0.008) for serum CRP as a categorical variable and an AUC of 0.59 (95% CI 0.52 – 0.66, p=0.015) for CRP as a continuous variable (figure 3.39).

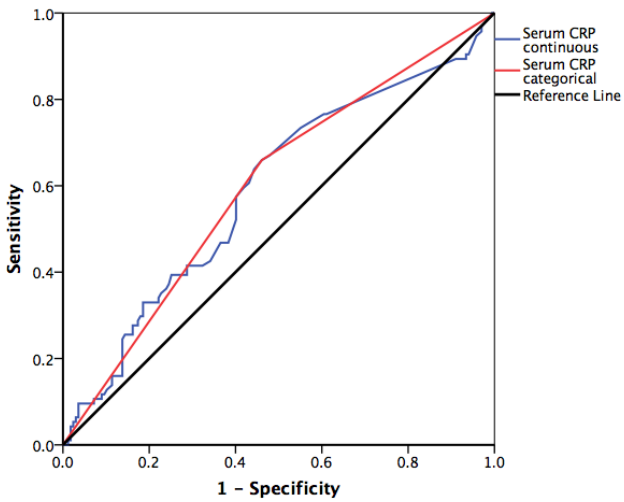


Figure 3.39: The predictive value of CRP in identifying patients with stage II colorectal cancer who will die during follow-up

Receiver-operator-characteristic curve demonstrating the predictive value of serum CRP in identifying patients who will die during follow-up.

Univariable overall survival - serum albumin

Serum albumin measurements were significantly lower in patients who died during the follow up phase (p<0.001, Mann-Whitney U, figure 3.40). Median serum albumin was 40 g/l (IQR 32 – 48 g/l) in the alive group compared to 37 g/l (IQR 28 – 46 g/l) in patients who did not survive 5 years.

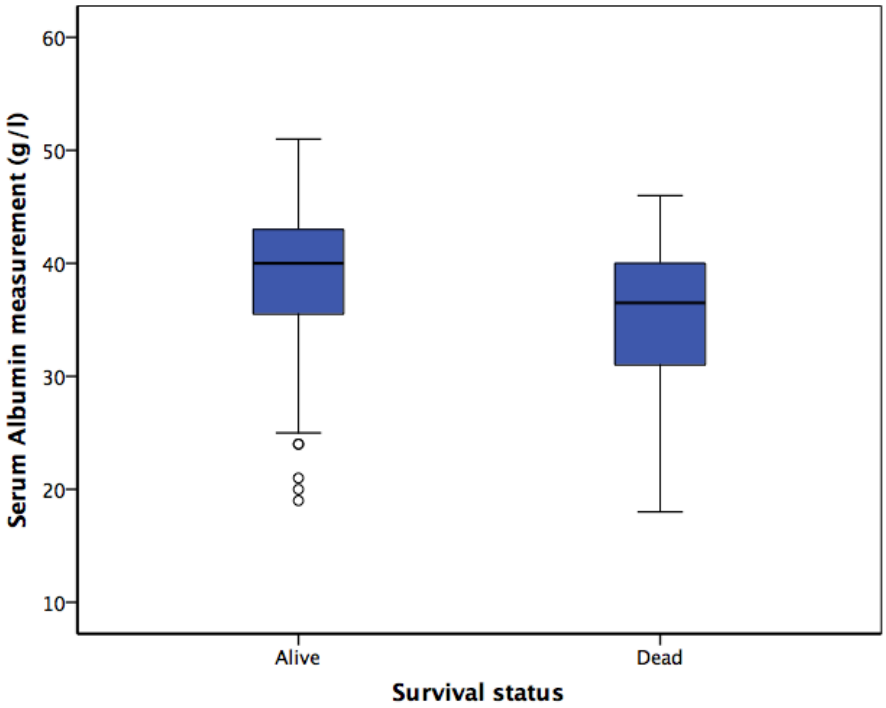
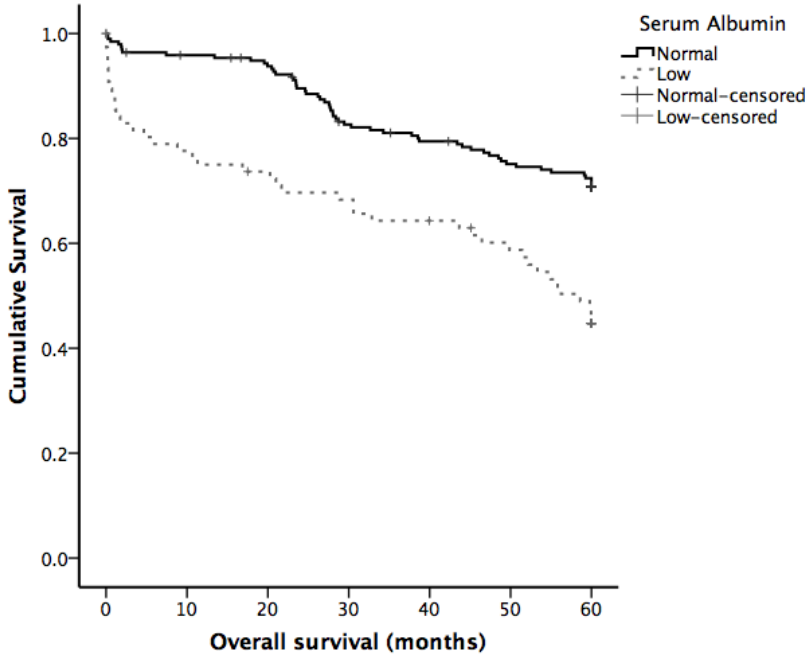


Figure 3.40: The distribution of serum albumin measurements in patients stratified by survival status (p<0.001)

The 5 year overall survival rate for patients with hypoalbuminaemia was 42.3% compared to 69.1% in patients with a normal serum albumin (p<0.001, Pearson's chi square). On logrank analysis hypoalbuminaemia was significantly associated with poor overall survival (p<0.001, figure 3.41). The mean survival for patients with hypoalbuminaemia was 40.7 months (95% CI 35.1 – 46.2) compared with 51.4 months (95% CI 49.1 – 53.7) in the normal serum albumin group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	194	184	178	155	148	139	134
Low	78	59	55	51	48	43	33

Figure 3.41: The relationship between serum albumin expression and overall survival in patients with stage II colorectal cancer

Kaplan-Meier curve demonstrating the proportion of patients surviving stratified by serum albumin expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis hypoalbuminaemia was significantly associated with poorer overall survival when dichotomised as a categorical variable (HR 2.37 (95% CI 1.58 – 3.55), p<0.001). Hypoalbuminaemia was also significantly associated with survival when included as a continuous variable (HR 0.94 (95% CI 0.91 – 0.96), p<0.001). The results of the Kaplan-Meier plot, however, suggest that serum albumin violates the proportionality assumption of the Cox model. There appears to be a greater proportion of events in the low group near the origin of the curve. The subsequent gradient associated with these events does not hold throughout the follow-up period suggesting that the hazard ratio is not constant over time thus violating the proportionality assumption. When assessing the log(-log(survival)) plot (figure 3.42), categorical serum albumin demonstrates parallel curves, however, there is evidence of time dependency when an interaction term between serum Albumin and log(time) was placed in a Cox proportional hazard model (HR 0.76 (95% CI 0.60 – 0.96); p=0.023) with an associated adjustment of the HR for categorical serum albumin (HR 4.73 (95% CI 2.25 – 9.96); p=<0.001).

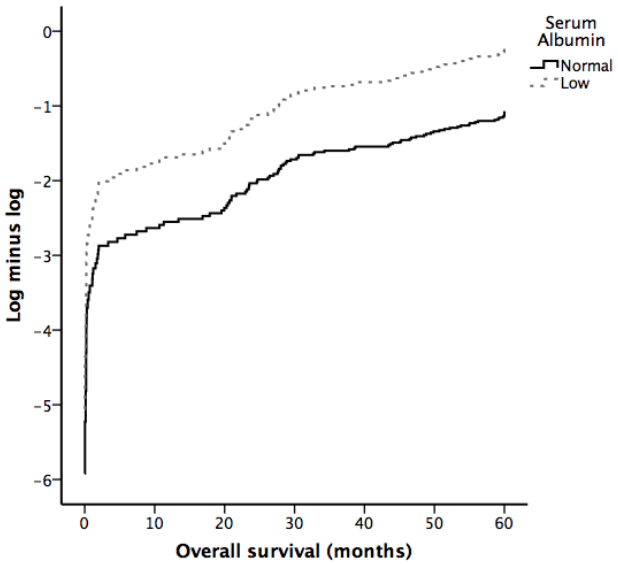


Figure 3.42: Log minus log plot of serum albumin and overall survival in patients with stage II colorectal cancer

Log(-log(survival)) plot demonstrating near proportionality of survival between the two groups.

In terms of survival prediction, when ROC analysis was performed using death as the endpoint, the AUC was 0.35 (95% CI 0.28 – 0.42, p<0.001) for albumin as a continuous variable and an AUC of 0.61 (95% CI 0.54 – 0.68, p=0.003) for albumin as a categorical variable (figure 3.43).

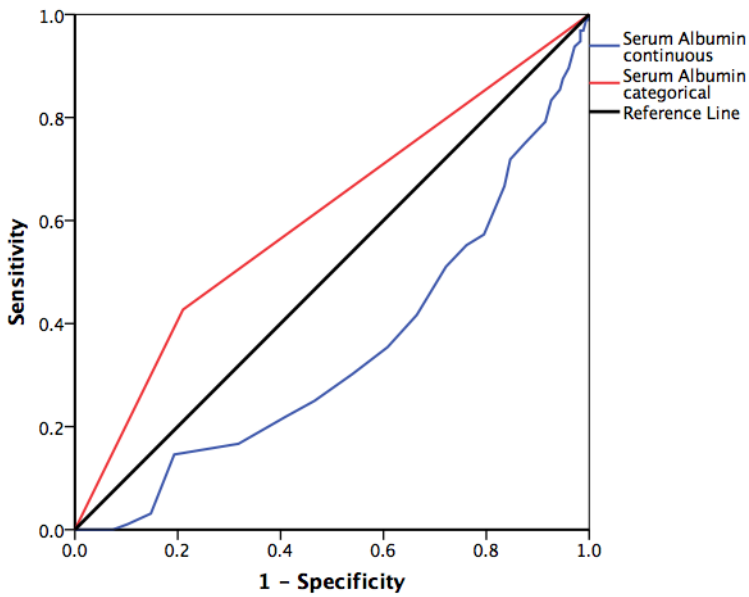


Figure 3.43: Predictive value of serum albumin in identifying patients with stage II colorectal cancer who will die during follow-up

Receiver-operator-characteristic curve demonstrating the predictive value of serum albumin in identifying patients who will die during follow-up.

Univariable overall survival - Tumour MSI status

Of the patients with MSI tumours, 106 (32.%) died during follow-up compared with 98 (35.4%) of the patients with CI tumours (p=0.748, Fisher's exact test). The 5 year survival rate for patients with MSI tumours was 60.0% compared to 61.0% compared to patients with CI tumours (p=0.877, Pearson's chi square). On logrank analysis, MSI status was not associated with overall survival (p=0.688). The mean survival for patients with MSI tumours was 49.2 months (95% CI 43.9 – 54.6) compared with 48.2 months (95% CI 45.8 – 50.5) in the CI group (supplementary figure 3.19, appendix 5.1).

On Cox univariable regression analysis MSI status was not significantly associated with improved overall survival (HR 0.90 (95% CI 0.53 – 1.52), p=0.688). Furthermore, MSI status did not significantly predict death during the follow up period with an AUC of 0.49 (95% CI 0.43 – 0.56, p=0.774).

Multivariable recurrence-free survival

Proportionality assumptions testing

All the studied variables underwent Cox proportional hazards assumption testing as described in the statistical methodology section 2.7.8.7.

Cox proportional hazards regression analysis

All covariates with a significance level of p<0.1 were included in the multivariable model. Terms were removed if their respective p-values were >0.05. On univariable analysis, advancing age (p=0.096), emergency presentation (p=0.015), advancing T-stage (p<0.001), raised serum CRP (p=0.068), and tumour MSI status (p=0.090) had a p-value <0.1 and were therefore included in the multivariable Cox proportional hazards regression model.

On multivariable analysis, advancing T-stage (HR 2.99 (95% CI 1.65 – 5.40), p<0.001) and emergency presentation (HR 1.89 (95% CI 1.65 – 3.43), p=0.035) were independently and significantly associated with poor recurrence-free survival (table 3.12).

Table 3.12: The relationships between clinicopathological factors and recurrence-free survival in patients with stage II colorectal cancer – univariable and multivariable analysis

	Univariable analysis			Multivariable analysis		
	Coefficient	p-value	Hazard ratio (95% CI)	Coefficient	p-value	Hazard ratio (95% CI)
Age	-0.020	0.096	0.98 (0.96 - 1.00)		0.325	
Sex						
Female			1			
Male	0.243	0.376	1.28 (0.74 – 2.19)			
Presentation						
Elective			1			1
Emergency	0.671	0.015	1.96 (1.14 – 3.36)	0.638	0.035	1.89 (1.05 – 3.43)
Tumour site						
Right	0.000	0.533	1			
Left	0.250		1.28 (0.70 – 2.37)			
Rectum	0.373		1.45 (0.73 – 2.90)			
T stage						
1	N/A		N/A	N/A		N/A
2	N/A		N/A	N/A		N/A
3	(0.000)		1	(0.000)		1
4	0.973	<0.001	2.65 (1.55 – 4.51)	1.094	<0.001	2.99 (1.65 – 5.40)
Differentiation						
Well/Moderate			1			
Poor	0.037	0.938	1.04 (0.41 – 2.60)			
Serum CRP						
Normal			1			
High	0.561	0.068	1.75 (0.96 – 3.20)		0.422	
Serum albumin						
Normal			1			
Low	0.298	0.350	1.35 (0.72 – 2.52)			
Klintrup score						
High			1			
Low	-0.471	0.150	0.62 (0.33 – 1.19)			
MSI status						
CI			1			
MSI	-0.876	0.092	0.42 (0.15 – 1.15)		0.184	

Multivariable overall survival

Proportionality assumptions testing

All the studied variables underwent Cox proportional hazards assumption testing as described in the statistical methodology section 2.7.8.7. Presentation, serum CRP and Serum albumin demonstrated time dependency when interaction terms between the covariates and log(time) were placed in the model.

Cox proportional hazards regression analysis

All covariates with a significance level of $p<0.1$ were included in the multivariable model. Terms were removed if their respective p-value was >0.05. On univariable analysis,

advancing age (p=0.001), emergency presentation (p<0.001), advancing T-stage (p=0.003), raised serum CRP (p=0.002), hypoalbuminaemia (p<0.001) and good Klintrup score (p=0.022) had a p-value <0.1 and were therefore included in the multivariable Cox proportional hazards regression model.

On multivariable analysis, only emergency presentation (HR 5.74 (95% CI 1.04 – 14.68), p<0.001), advancing age (HR 1.04 (95% CI 1.01 – 1.06), p=0.002), hypoalbuminaemia (HR 3.04 (95% CI 1.44 – 6.43), p=0.004), advancing T-stage (HR 1.70 (95% CI 1.11 – 2.58), p=0.014) and Klintrup score (HR 0.60 (95% CI 0.37 – 0.97), p=0.039) were independently and significantly associated with poor overall survival (table 3.13).

Influence of time dependency on outcome

Only emergency presentation (p=0.004) retained significant time dependency when placed in the multivariable analysis with serum CRP (p=0.360) and serum albumin (p=0.096) becoming non-significant.

Table 3.13: The relationships between clinicopathological factors and overall survival in patients with stage II colorectal cancer – univariable and multivariable analysis

	Univariable analysis			Multivariable analysis		
	Coefficient	p-value	Hazard ratio (95% CI)	Coefficient	p-value	Hazard ratio (95% CI)
Age	0.033	0.001	1.03 (1.01 - 1.05)	0.035	0.002	1.04 (1.01 – 1.06)
Sex						
Female			1			
Male	0.136	0.468	1.15 (0.79 – 1.66)			
Presentation						
Elective			1			1
Emergency	1.548	<0.001	4.70 (2.29 – 9.65)	1.747	<0.001	5.74 (2.24 – 14.68)
Presentation x log(time)	-0.320	0.006	0.73 (0.58 – 0.91)	-0.428	0.004	0.65 (0.49 – 0.88)
Tumour site						
Right	(0.000)	0.539	1			
Left	0.180		1.20 (0.80 – 1.79)			
Rectum	-0.084		0.92 (0.55 – 1.55)			
T stage						
1	N/A		N/A	N/A		N/A
2	N/A		N/A	N/A		N/A
3	(0.000)		1	(0.000)		1
4	0.292	0.003	1.78 (1.22 – 2.58)	0.528	0.014	1.70 (1.11 – 2.58)
Differentiation						
Well/Moderate			1			
Poor	0.137	0.666	1.15 (0.62 – 2.14)			
Serum CRP						
Normal			1			
High	1.617	0.002	5.04 (1.78 – 14.24)		0.137	
CRP x log(time)	-0.313	0.051	0.73 (0.54 – 1.00)		0.360	
Serum albumin						
Normal			1			1
Low	1.554	<0.001	4.73 (2.25 – 9.96)	1.111	0.004	3.04 (1.44– 6.43)
albumin x log(time)	-0.275	0.023	0.76 (0.60 – 0.96)	-0.202	0.096	0.82 (0.84 – 1.04)
Klintrup score						
High			1			1
Low	-0.525	0.022	0.59 (0.38 – 0.93)	-0.511	0.039	0.60 (0.37 – 0.97)
MSI status						
CI			1			
MSI	-0.108	0.688	0.90 (0.53 – 1.52)			

3.4.5.7 The association of serum CRP, albumin, MSI status and survival stratified by TNM stage III

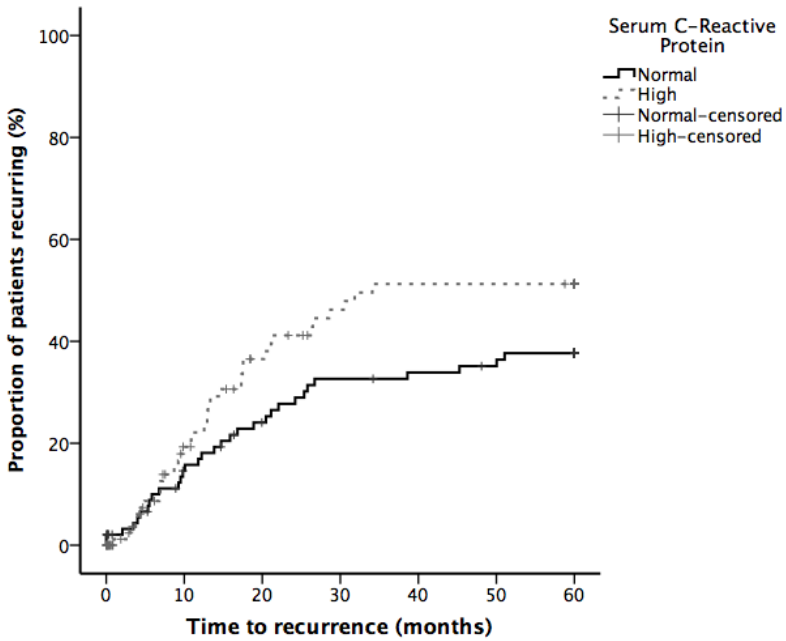
During follow-up there were 89 (36.3%) recurrences and 121 (49.4%) deaths. Five year recurrence-free and overall survival rates for the clinicopathological factors studied can be found in appendix 3.4. Based on a 15% difference in 5 year survival with an α=0.05 and β=0.2 the number of patient required was n=338. For a multivariable model containing at

least 10 variables a minimum of 100 events are required. Only 55% power was available when TNM stage III disease was studied.

Univariable recurrence-free survival – serum CRP expression

Serum CRP measurements were not significantly different in patients who went on to develop disease recurrence (p=0.557, Mann-Whitney U, supplementary figure 3.20, appendix 5.1). The median for patients with recurrence was 12.50 mg/l (IQR 3.00 – 41.75 mg/l) compared with 10.00 mg/l (IQR 1.00 – 44.50 mg/l) in the non-recurrence group.

The 5 year recurrence free survival rate for patients with a raised serum CRP was 28.9% compared to 51.0% in patients with a normal serum CRP (p=0.002, Pearson's chi square). On logrank analysis raised serum CRP was not significantly associated with poor recurrence-free survival (p=0.093, figure 3.44). The mean survival for patients with a raised serum CRP was 36.8 months (95% CI 31.3 – 42.3) compared with 43.9 months (95% CI 39.1 – 48.6) in the normal serum CRP group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	96	73	62	54	52	51	49
High	97	57	41	32	29	29	28

Figure 3.44: The relationship between serum CRP expression and recurrence-free survival in patients with stage III colorectal cancer

Kaplan-Meier curve demonstrating the proportion of patients recurring stratified by serum CRP expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

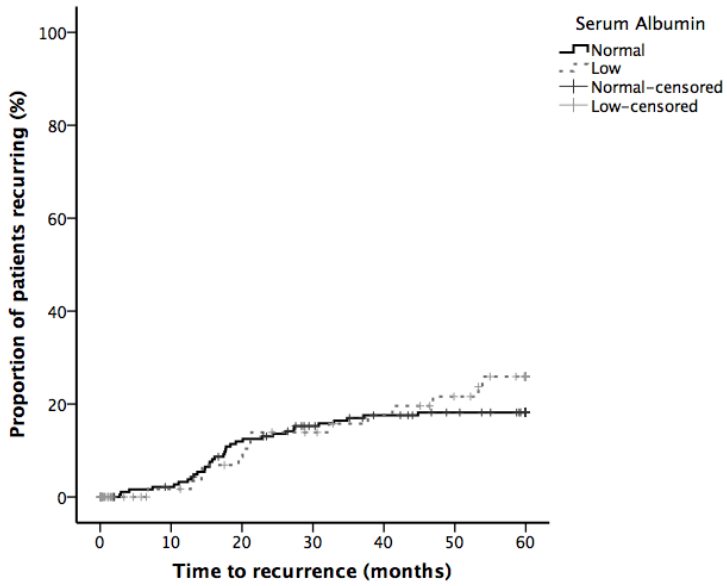
Similar to logrank analysis, on Cox univariable regression analysis raised serum CRP was not significantly associated with poor recurrence-free survival when dichotomised as a categorical variable (HR 1.50 (95% CI 0.93 – 2.42), p=0.095). The hazard ratio was less significant when included as a continuous variable (HR 1.00 (95% CI 1.00 – 1.01), p=0.401).

In addition to the prognostic observations with Cox proportional hazards test, the predictive value of serum CRP in identifying patients who developed recurrence during follow-up was also poor. When ROC analysis was performed using recurrence as the endpoint, the AUC was 0.53 (95% CI 0.44 – 0.61, p=0.560) for CRP as a continuous variable and the AUC was 0.52 (95% CI 0.44 – 0.61, p=0.635) for CRP as a categorical variable (supplementary figure 3.21, appendix 5.1).

Univariable recurrence-free survival – serum albumin

There was no statistically significant difference between the serum albumin measurements of patients who did and did not develop disease recurrence (p=0.256, Mann-Whitney U, (supplementary figure 3.22, appendix 5.1). The median was 39 g/l (IQR 31 – 47 g/l) in the non-recurrence group compared to 37 g/l (IQR 27.25 – 46.75 g/l) in the recurrence group.

The 5 year recurrence-free survival rate for patients with hypoalbuminaemia was 19.3% compared to 48.0% in patients with a normal serum albumin (p<0.001, Pearson's chi square). On logrank analysis hypoalbuminaemia was associated with poor recurrence-free survival (p=0.007, figure 3.45). The mean survival for patients with hypoalbuminaemia was 32.7 months (95% CI 25.3 – 40.1) compared with 43.5 months (95% CI 39.6 – 47.3) in the normal serum albumin group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	148	112	95	79	75	73	70
Low	57	30	17	15	13	13	11

Figure 3.45: The relationship between serum albumin expression and recurrence free survival in patients with stage III colorectal cancer (p=0.007)

Kaplan-Meier curve demonstrating the proportion of patients recurring stratified by serum albumin expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis hypoalbuminaemia was significantly associated with poor recurrence-free survival when dichotomised as a categorical variable (HR 1.95 (95% CI 1.20 – 3.20), p=0.008). The hazard ratio was also significant when analysed as a continuous variable (HR 0.95 (95% CI 0.93 – 0.98), p=0.002). There was no statistically significant predictive association between serum albumin and disease recurrence when analysed using ROC analysis. With disease recurrence as the endpoint, the AUC was 0.45 (95% CI 0.37 – 0.54, p=0.257) for albumin as a categorical variable and AUC 0.54 (95% CI 0.46 – 0.62, p=0.351) for albumin as a continuous variable (supplementary figure 3.23, appendix 5.1).

Univariable recurrence-free survival – tumour MSI status

Of the patients with MSI tumours, 5 (23.8%) developed cancer recurrence compared with 84 (37.5%) of the patients with CI tumours (p=0.244, Fisher's exact test). The 5 year recurrence free survival rate for patients with MSI tumours was 19% compared to 40.2% in patients with CI tumours (p=0.063, Pearson's chi square). On logrank analysis, MSI status was not significantly associated with improved recurrence-free survival (p=0.836). The

mean survival for patients with MSI tumours was 40.6 months (95% CI 27.6 – 53.7) compared with 40.9 months (95% CI 37.6 – 44.1) in the CI group (supplementary figure 3.24, appendix 7.1).

On Cox univariable regression analysis, MSI tumours were not significantly associated with improved recurrence-free survival (HR 0.91 (95% CI 0.37 – 2.25), p=0.836), furthermore, MSI status did not significantly predict the development of recurrence with an AUC of 0.48 (95% CI 0.40 – 0.55, p=0.546)

Univariable overall survival - serum CRP expression

Serum CRP measurements were significantly higher in patients who died during follow-up (p<0.001, Mann-Whitney U, figure 3.46). The median for patients who died was 16.50 mg/l (IQR 3.00 – 62.00 mg/l) compared with 7.00 mg/l (IQR 1.00 – 26.00 mg/l) in the alive group.

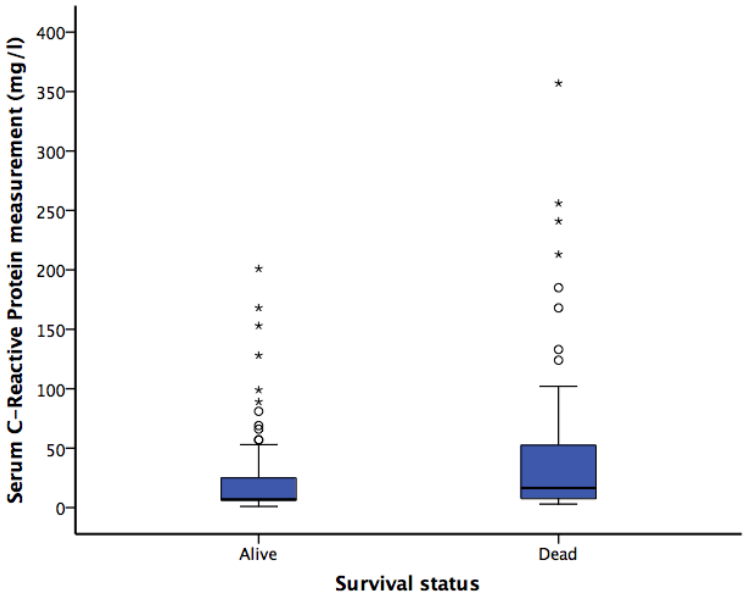
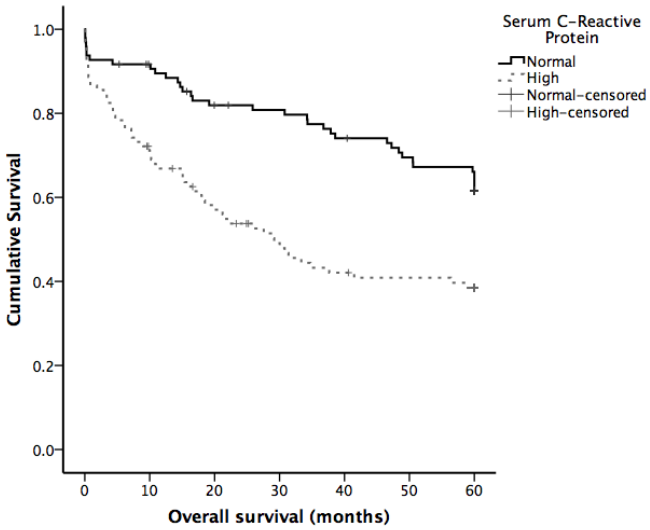


Figure 3.46: The distribution of serum CRP measurements in patients stratified by survival status (p<0.001)

The 5 year overall survival rate for patients with a raised serum CRP was 33.0% compared to 59.4% in patients with a normal serum CRP (p<0.001, Pearson's chi square). On logrank analysis raised serum CRP was significantly associated with poor overall survival (p<0.001, figure 3.47). The mean survival for patients with a raised serum CRP was 32.3 months (95% CI 27.3 – 37.3) compared with 47.8 months (95% CI 43.6 – 52.0) in the normal serum CRP group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	96	85	74	72	66	61	58
High	97	67	52	42	36	34	32

Figure 3.47: The relationship between serum CRP expression and overall survival in patients with stage III colorectal cancer (p<0.001)

Kaplan-Meier curve demonstrating the proportion of patients surviving stratified by serum CRP expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis raised serum CRP was significantly associated with poor overall survival when dichotomised as a categorical variable (HR 2.21 (95% CI 1.45 – 3.38), p<0.001). Serum CRP was also associated with poor overall survival when analysed as a continuous variable (HR 1.01 95% CI 1.00 – 1.01, p<0.001). The Kaplan-Meier plot, however, suggests that serum CRP may violate the proportionality assumption of the Cox proportional hazards model. There appears to be a greater proportion of events in the high expression group near the origin of the curve. The subsequent gradient associated with these events does not appear to hold throughout the follow up period suggesting that the HR is not constant thus potentially violating the proportionality assumption. The log(-log(survival)) plot (figure 3.48), of categorical serum CRP demonstrates parallel curves with no evidence of time dependency when an interaction term between serum CRP and log(time) was placed in a Cox proportional hazard model (HR 0.95 (95% CI 0.76 – 1.19); p=0.636). These findings suggest that serum CRP does in fact meet the proportionality assumptions of the Cox proportional hazards regression model.

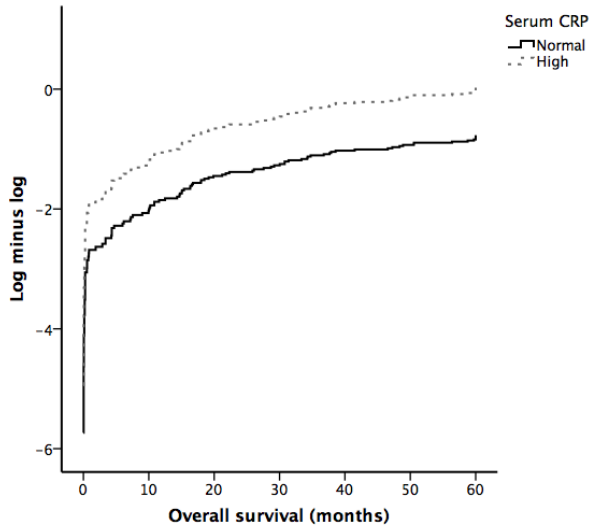


Figure 3.48: Log minus log plot of serum CRP and overall survival

Log(-log(survival)) plot demonstrating near proportionality of survival between the two groups

Despite the significant associations observed between serum CRP and survival, its predictive value is relatively poor. When ROC analysis was performed using death as the endpoint, the AUC was 0.61 (95% CI 0.53 – 0.69, p=0.007) for serum CRP as a categorical variable and the AUC was 0.65 (95% CI 0.58 – 0.73, p<0.001) for CRP as a continuous variable (figure 3.49).

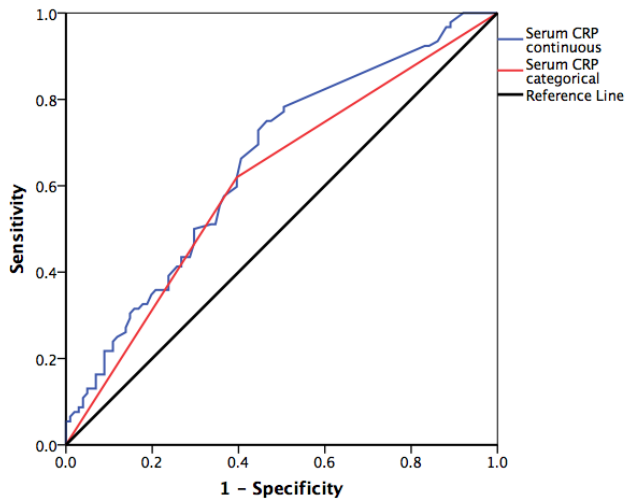


Figure 3.49: Predictive value of CRP in identifying patients with stage III colorectal cancer who will die during follow-up

Receiver-operator-characteristic curve demonstrating the predictive value of serum CRP in identifying patients who will die during follo- up.

Univariable overall survival - serum albumin

Serum albumin measurements were significantly lower in patients who died during follow-up (p=0.002, Mann-Whitney U, figure 3.50). The median was 40 g/l (IQR 33 – 47 g/l) in the alive group compared to 37 g/l (IQR 26 – 48 g/l) in deceased group.

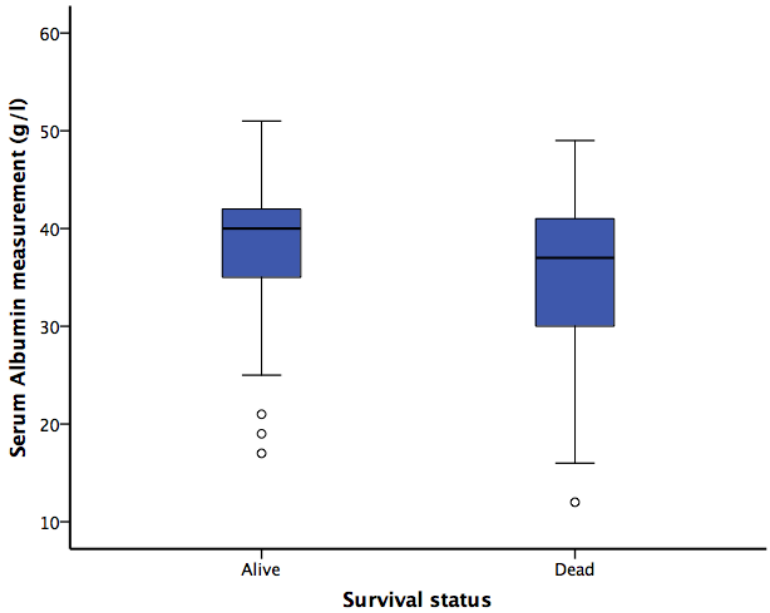
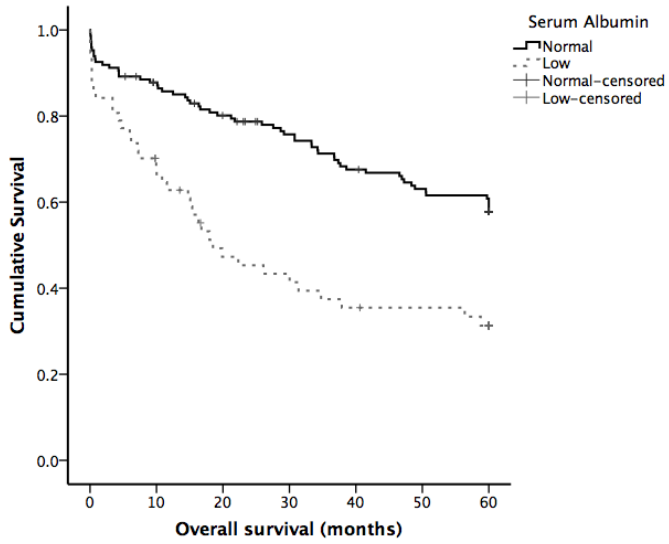


Figure 3.50: The distribution of serum albumin measurements in patients stratified by survival status (p=0.002)

The 5 year overall survival rate for patients with hypoalbuminaemia was 26.3% compared to 54.1% in patients with a normal serum albumin (p<0.001, Pearson's chi square). On logrank analysis hypoalbuminaemia was significantly associated with poor overall survival (p<0.001, figure 3.51). The mean survival for patients with hypoalbuminaemia was 29.0 months (95% CI 22.5 – 35.5) compared with 45.1 months (95% CI 41.6 – 48.7) in the normal serum albumin group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	148	126	113	102	91	84	81
Low	57	37	24	22	18	17	15

Figure 3.51: The relationship between serum albumin expression and overall survival in patients with stage III colorectal cancer (p<0.001)

Kaplan-Meier curve demonstrating the proportion of patients surviving stratified by serum albumin expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis hypoalbuminaemia was significantly associated with poor overall survival when dichotomised as a categorical variable (HR 2.36 (95% CI 1.56 – 3.57), p<0.001). The hazard ratio was also significantly associated with survival when included as a continuous variable (HR 0.95 (95% CI 0.91 – 0.73), p<0.001). The Kaplan-Meier plot, however, suggest that serum albumin may violate the proportionality assumption of the Cox model, with a greater proportion of events in the low expression group near the origin of the curve. The gradient associated with these events is not maintained throughout follow-up which suggests that the hazard ratio is not constant, violating the proportionality assumption. However, the log(-log(survival)) plot (figure 3.52), for categorical serum albumin demonstrates parallel curves with no evidence of time dependency when an interaction term between serum albumin and log(time) was placed in a Cox proportional hazard model (HR 0.98 (95% CI 0.79 – 1.20); p=0.809). These findings suggest that serum albumin does in fact meet the proportionality assumptions of the Cox proportional hazards regression model.

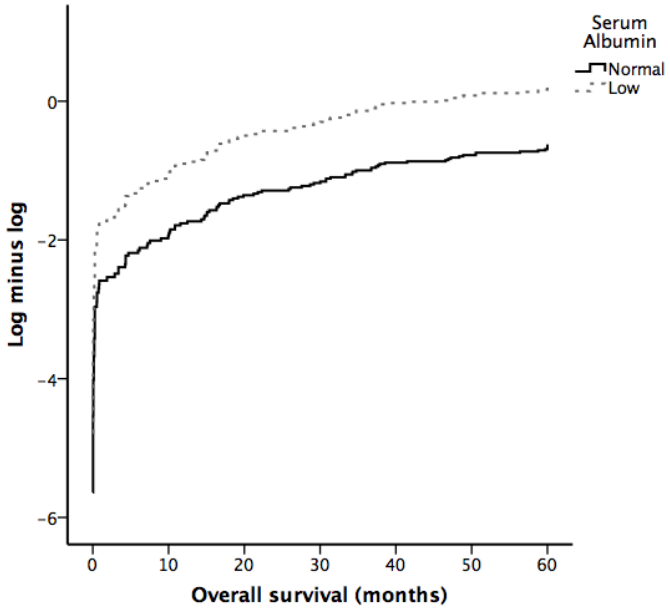


Figure 3.52: Log minus log plot of serum albumin and overall survival in patients with stage III colorectal cancer

Log(-log(survival)) plot demonstrating near proportionality of survival between the two groups.

In terms of survival prediction, when ROC analysis was performed using death as the endpoint, the AUC was 0.38 (95% CI 0.30 – 0.45, p=0.002) for albumin as a continuous variable and an AUC of 0.60 (95% CI 0.52 – 0.68, p=0.013) for albumin as a categorical variable (figure 3.53).

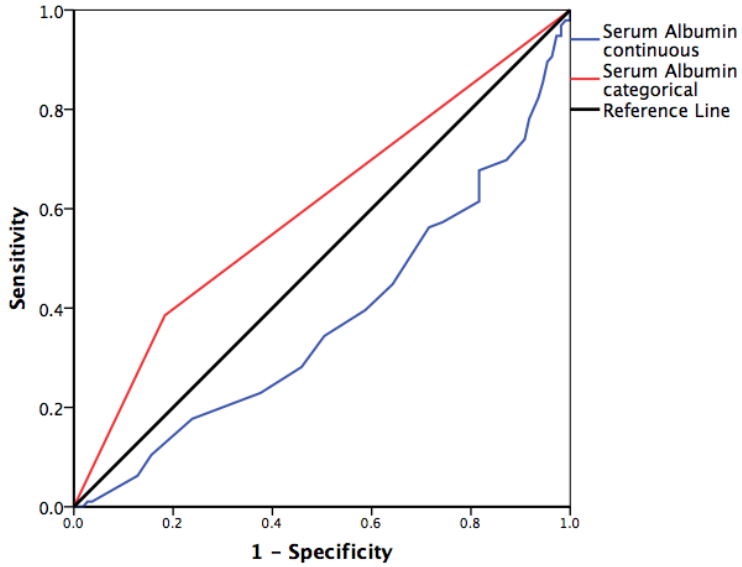
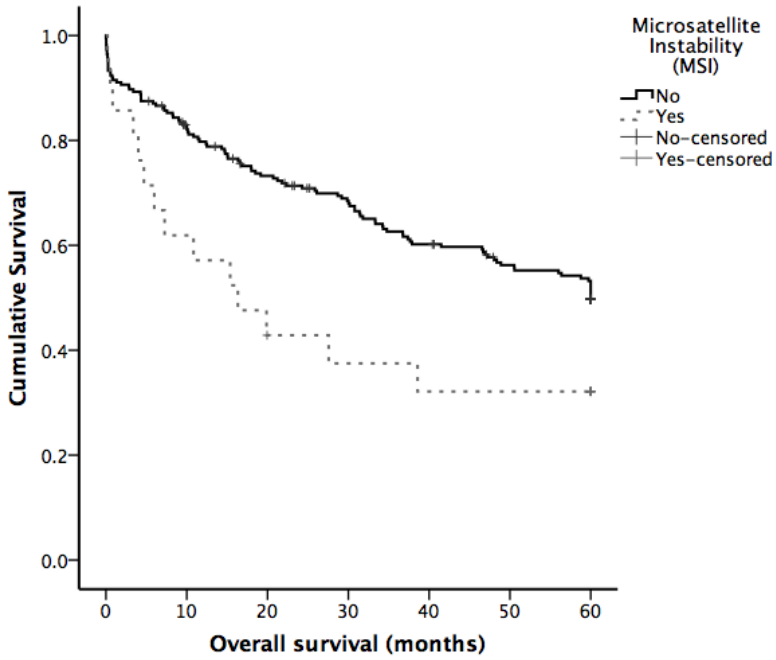


Figure 3.53: Predictive value of serum albumin in identifying patients with stage III colorectal cancer who will die during follow-up

Receiver-operator-characteristic curve demonstrating the predictive value of serum albumin in identifying patients who will die during follow-up.

Univariable overall survival - tumour MSI status

Of the patients with MSI tumours, 14 (66.7%) died during follow-up compared with 107 (47.8%) of the patients with CI tumours (p=0.113, Fisher's exact test). The 5 year survival rate for patients with MSI tumours was 28.6% compared to 46.9% compared to patients with CI tumours (p=0.107, Pearson's chi square). On logrank analysis, MSI status was associated with poor overall survival (p=0.020, figure 3.54). The mean survival for patients with MSI tumours was 27.1 months (95% CI 16.5 – 37.6) compared with 41.3 months (95% CI 38.2 – 44.4) in the CI group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
CI	224	179	156	141	124	112	106
MSI	21	13	8	7	6	6	6

Figure 3.54: The relationship between MSI status and overall survival in patients with stage III colorectal cancer (p=0.020)

Kaplan-Meier curve demonstrating the proportion of patients surviving stratified by MMR protein status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis MSI was significantly associated with poor overall survival (HR 1.91 (95% CI 1.10 – 3.35), p=0.023). However, MSI status did not significantly predict death during the follow up period with an AUC of 0.53 (95% CI 0.46 – 0.60, p=0.423).

Multivariable recurrence-free survival

Proportionality assumptions testing

All the studied variables underwent Cox proportional hazards assumption testing as described in the statistical methodology section 2.7.8.7.

Cox proportional hazards regression analysis

All covariates with a significance level of $p<0.1$ were included in the multivariable model. Terms were removed if their respective p-values were >0.05. On univariable analysis, emergency presentation (p=0.031), advancing T-stage (p=0.001), higher N stage (p=0.013), poor differentiation (p=0.026), raised serum CRP (p=0.095), hypoalbuminaemia (p=0.008) and a good Klintrup score (p=0.001) had p-values <0.1 and were therefore included in the multivariable Cox proportional hazards regression model.

On multivariable analysis, advancing T-stage (when all subcategories were compared with T1 (T2 HR 280.27 (95% CI 0.00 – 1.18e45), (T3 HR 278.42 (95% CI 0.00 – 1.16e45) and (T4 HR 683.12 (95% CI 0.00 – 2.85e45)), p=0.010), hypoalbuminaemia (HR 1.86 (95% CI 1.03 – 3.36), p=0.040) and a high Klintrup score (HR 0.40 (95% CI 0.18 – 0.91), p=0.028) were independently associated with recurrence-free survival (table 3.14).

Table 3.14: The relationship between clinicopathological factors and recurrence-free survival in patients with stage III colorectal cancer – univariable and multivariable analysis

	Univariable analysis			Multivariable analysis		
	Coefficient	p-value	Hazard ratio (95% CI)	Coefficient	p-value	Hazard ratio (95% CI)
Age	-0.002	0.834	1.00 (0.98 - 1.02)			
Sex						
Female			1			
Male	-0.092	0.665	1.10 (0.72 – 1.66)			
Presentation						
Elective			1			
Emergency	0.472	0.031	1.60 (1.04 – 2.47)		0.852	
Tumour site						
Right	0.000	0.102	1			
Left	-0.496		0.61 (0.37 – 1.00)			
Rectum	0.065		1.07 (0.63 – 1.82)			
T stage						
1	(0.000)	0.001	1	(0.000)	0.010	1
2	5.972		392.11 (0.00 – 1.18e42)	5.746		280.27 (0.00 – 1.18e45)
3	6.852		945.66 (0.00 – 2.82e42)	5.659		278.42 (0.00 – 1.16e45)
4	7.666		2134.90 (0.00 – 6.37e42)	6.574		683.12 (0.00 – 2.85e45)
N stage						
1	(0.000)		1			
2	0.547	0.013	1.73 (1.12 – 2.69)		0.192	
Differentiation						
Well/Moderate			1			
Poor	0.617	0.026	1.85 (1.08 – 3.19)		0.393	
Serum CRP						
Normal			1			
High	0.407	0.095	1.50 (0.93 – 2.42)		0.321	
Serum albumin						
Normal			1			1
Low	0.670	0.008	1.95 (1.20 – 3.20)	0.620	0.040	1.86 (1.03 – 3.36)
Klintrup score						
High			1			1
Low	-1.257	0.001	0.28 (0.14 – 0.59)	-0.914	0.028	0.40 (0.18 – 0.91)
MSI status						
CI			1			
MSI	-0.096	0.836	0.91 (0.37 – 2.25)			

Multivariable overall survival

Proportionality assumptions testing

All the studied variables underwent Cox proportional hazards assumption testing as described in the statistical methodology section 2.7.8.7.

Cox proportional hazards regression analysis

All covariates with a significance level of $p<0.1$ were included in the multivariable model. Terms were removed if their respective p-values were >0.05. On univariable analysis, advancing age ($p<0.001$), emergency presentation ($p<0.001$), tumour location ($p=0.067$),

advancing T-stage (p=0.009), advancing N-stage (p=0.019), poor differentiation (p=0.001), raised serum CRP (p<0.001), hypoalbuminaemia (p<0.001), good Klintrup score (p=0.002) and tumour MSI status (p=0.023) had a p-value <0.1 and were therefore included in the multivariable Cox proportional hazards regression model.

On multivariable analysis, only advancing age (HR 1.04 (95% CI 1.02 – 1.07), p<0.001), emergency presentation (HR 1.81 (95% CI 1.06 – 3.06), p=0.028), tumour site (when all subcategories were compared with right sided location (Left sided cancers (HR 0.71 (95% CI 0.43 – 1.19) and rectal cancer (HR 1.75 (95% CI 0.99 – 3.10), p=0.027), good Klintrup score (HR 0.47 (95% CI 0.24 – 0.94), p=0.033) and MSI status (HR 2.20 (95% CI 1.10 – 4.37), p=0.025) were independently and significantly associated with poor overall survival following (table 3.15).

Table 3.15: The relationship between clinicopathological factors and overall survival in patients with stage III colorectal cancer: univariable and multivariable analysis

	Univariable analysis			Multivariable analysis		
	Coefficient	p-value	Hazard ratio (95% CI)	Coefficient	p-value	Hazard ratio (95% CI)
Age	0.037	<0.001	1.04 (1.02 - 1.06)	0.043	<0.001	1.04 (1.02 – 1.07)
Sex						
Female			1			
Male	-0.159	0.384	0.85 (0.60 – 1.22)			
Presentation						
Elective			1			1
Emergency	0.750	<0.001	2.12 (1.48 – 3.03)	0.591	0.028	1.81 (1.06 – 3.06)
Tumour site						
Right	(0.000)	0.067	1	(0.000)	0.027	1
Left	-0.499		0.61 (0.40 – 0.93)	-0.342		0.71 (0.43 – 1.19)
Rectum	-0.068		0.93 (0.59 – 1.49)	-0.562		1.75 (0.99 – 3.10)
T stage						
1	(0.000)	0.009	1		0.248	
2	6.392		596.78 (0.00 – 6.16e37)			
3	6.855		948.41 (0.00 – 9.73e37)			
4	7.421		1670.40 (0.00 – 1.71e38)			
N stage						
1			1			
2	0.954	0.019	1.56 (1.08 – 2.28)		0.389	
Differentiation						
Well/Moderate			1			
Poor	0.722	0.001	2.06 (1.32 – 3.20)		0.066	
Serum CRP						
Normal			1			
High	1.617	<0.001	2.21 (1.45 – 3.38)		0.937	
Serum albumin						
Normal			1			
Low	0.859	<0.001	2.36 (1.56 – 3.57)		0.059	
Klintrup score						
High			1			1
Low	-0.881	0.002	0.41 (0.24 – 0.72)	-0.746	0.033	0.47 (0.24 – 0.94)
MSI status						
CI			1			1
MSI	0.649	0.023	1.91 (1.10 – 3.35)	0.786	0.025	2.20 (1.10 – 4.37)

3.4.5.8 The association of systemic inflammation clinicopathological factors and survival CI colorectal cancer

Survival, recurrence and deaths in patients with CI colorectal cancer

During follow-up there were 140 (23.6%) recurrences and 227 (38.3%) deaths. Five year recurrence free and overall survival rates for the clinicopathological factors studied can be found in appendix 3.5. Based on a 15% difference in 5 year survival with an α=0.05 and β=0.2 the number of patients required was n=338. For a multivariable model containing at least 10 variables a minimum of 100 events are required.

Univariable recurrence-free survival - serum CRP expression

Serum CRP measurements were not significantly different in patients who went on to develop disease recurrence (p=0.067, Mann-Whitney U, figure 3.55). Median measurements for patients with recurrence was 12.00 mg/l (IQR 2.00 – 44.50 mg/l) compared with 9.00 mg/l (IQR 0.90 – 30.00 mg/l) in the non-recurrence group.

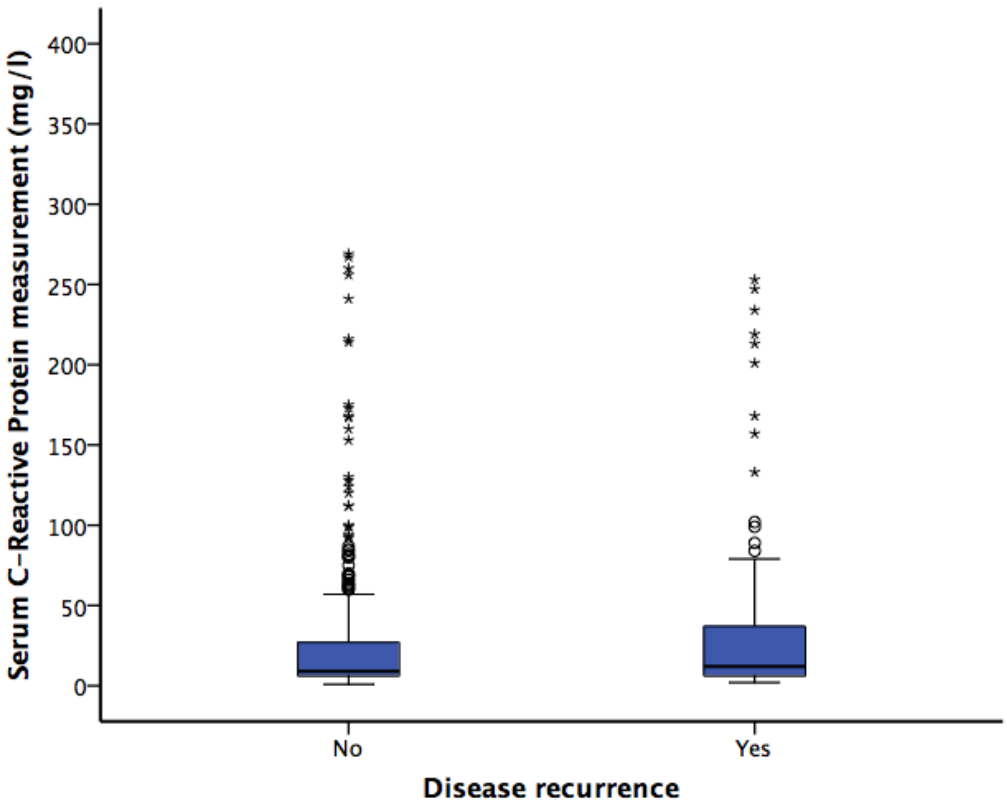
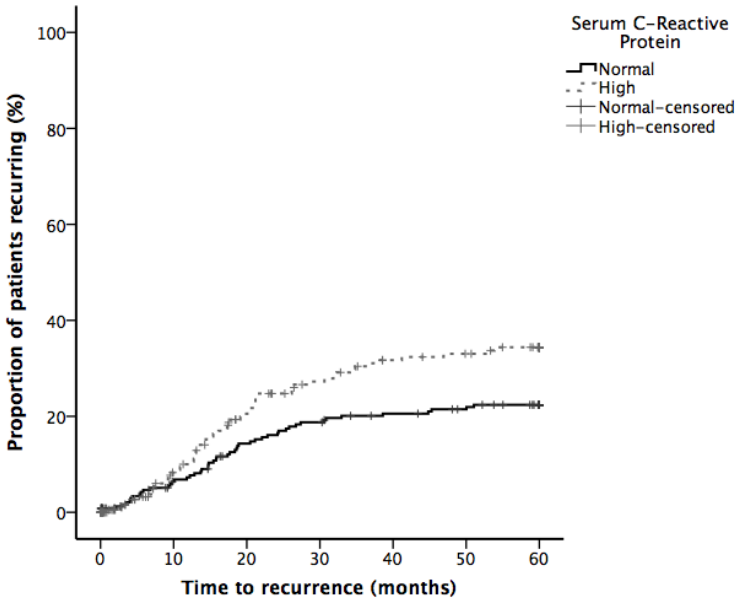


Figure 3.55: The distribution of serum CRP measurements in patients with and without cancer recurrence (p=0.067)

The 5 year recurrence-free survival rate for patients with a raised serum CRP was 42.0% compared to 64.9% in patients with a normal serum CRP (p<0.001, Pearson's chi square). On logrank analysis raised serum CRP was significantly associated with poor recurrence-

free survival (p=0.011, figure 3.56). The mean survival for patients with a raised serum CRP was 46.1 months (95% CI 43.0 – 49.1) compared with 50.6 months (95% CI 48.3 – 53.0) in the normal serum CRP group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	248	214	193	183	175	170	161
High	219	160	132	115	104	100	92

Figure 3.56: The relationship between serum CRP expression and recurrence-free survival in patients with CI colorectal cancer (p=0.011)

Kaplan-Meier curve demonstrating the proportion of patients recurring stratified by serum CRP expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis raised serum CRP was significantly associated with poor recurrence-free survival when dichotomised as a categorical variable (HR 1.62 (95% CI 1.11 – 2.36), p=0.012). The significance of the hazard ratio reduced when it was included as a continuous variable (HR 1.00 (95% CI 1.00 – 1.01), p=0.020). Despite the significant associations observed between serum CRP and disease recurrence, its predictive value remains poor. When ROC analysis was performed using recurrence as the endpoint, the AUC was 0.58 (95% CI 0.50 – 0.62, p=0.070) for CRP as a continuous variable with an AUC of 0.54 (95% CI 0.48 – 0.60, p=0.193) for CRP as a categorical variable (figure 3.57).

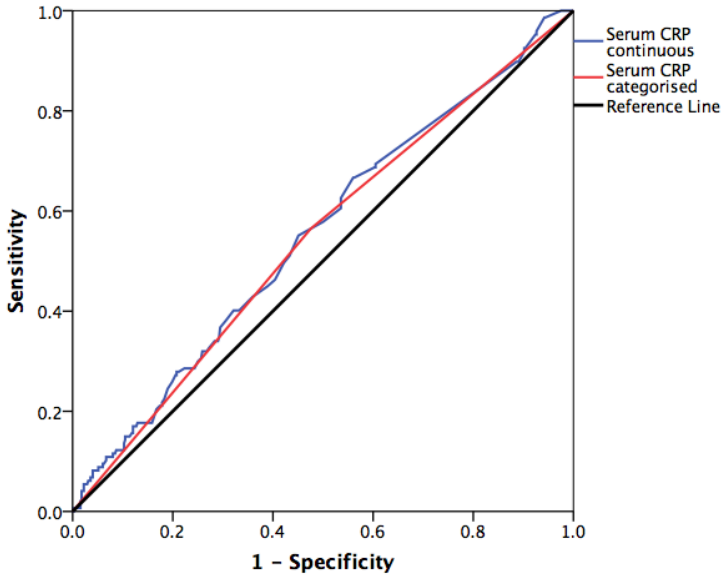


Figure 3.57: The predictive value of CRP in identifying patients with CI colorectal cancer who developed recurrence

Receiver-operator-characteristic curve demonstrating the predictive value of CRP in identifying patients who will develop disease recurrence.

Univariable recurrence-free survival - serum albumin

There was no statistically significant difference between the serum albumin measurements of patients who did and did not develop cancer recurrence (p=0.060, Mann-Whitney U, figure 3.58). The median was 39 g/l (IQR 30 – 48 g/l) in the non-recurrence group compared to 38 g/l (IQR 29 – 47 g/l) in the recurrence group.

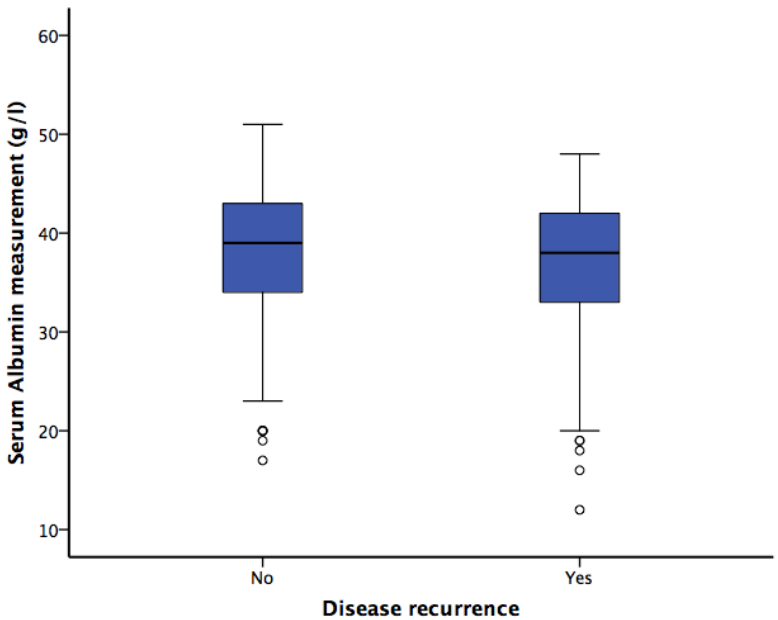
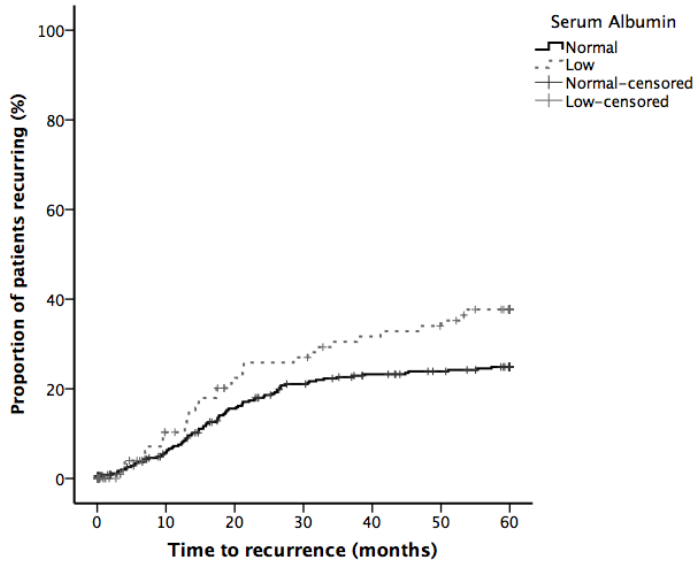


Figure 3.58: The distribution of serum albumin measurements in patients stratified by cancer recurrence (p=0.060)

The 5 year recurrence free survival rate for patients with hypoalbuminaemia was 36.2% compared to 60.7% in patients with a normal serum albumin (p<0.001, Pearson's chi square). On logrank analysis hypoalbuminaemia was associated with poorer time to recurrence (p=0.021, figure 3.59). The mean survival for patients with hypoalbuminaemia was 45.5 months (95% CI 41.3 – 49.8) compared with 49.7 months (95% CI 47.7 – 51.7) in the normal serum albumin group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	369	319	280	256	242	234	224
Low	127	83	69	64	58	55	46

Figure 3.59: The relationship between serum albumin expression and recurrence-free survival in patients with CI colorectal cancer (p=0.021)

Kaplan-Meier curve demonstrating the proportion of patients recurring stratified by serum albumin expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis hypoalbuminaemia was significantly associated with poor recurrence-free survival when dichotomised as a categorical variable (HR 1.59 (95% CI 1.07 – 2.38), p=0.022). The significance of the hazard ratio reduced when it was included as a continuous variable (HR 0.95 (95% CI 0.93 – 0.98), p<0.001). Despite the significant associations observed between serum albumin and disease recurrence, its predictive value is poor. When ROC analysis was performed using recurrence as the endpoint, the AUC was 0.44 (95% CI 0.38 – 0.50, p=0.060) for albumin as a continuous variable with an AUC of 0.52 (95% CI 0.46 – 0.58, p=0.459) for albumin as a categorical variable (figure 3.60).

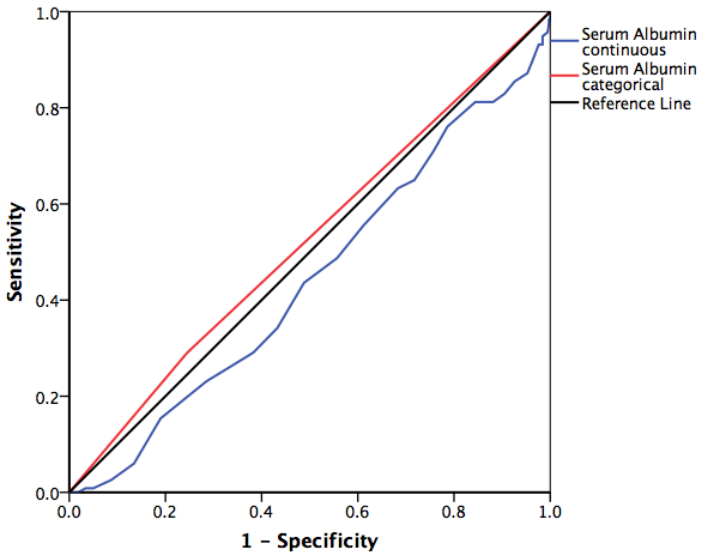


Figure 3.60: Predictive value of serum albumin in identifying patients who developed cancer recurrence

Receiver-operator-characteristic curve demonstrating the predictive value of serum albumin in identifying patients who will develop disease recurrence.

Univariable overall survival - serum CRP expression

Serum CRP measurements were significantly higher in patients who died during the follow up period ($p<0.001$, Mann-Whitney U, figure 3.61). The median for patients who died was 15.00 mg/l (IQR 1.00 – 56.75 mg/l) compared with 7.00 mg/l (IQR 0.90 – 22.00 mg/l) in the alive group.

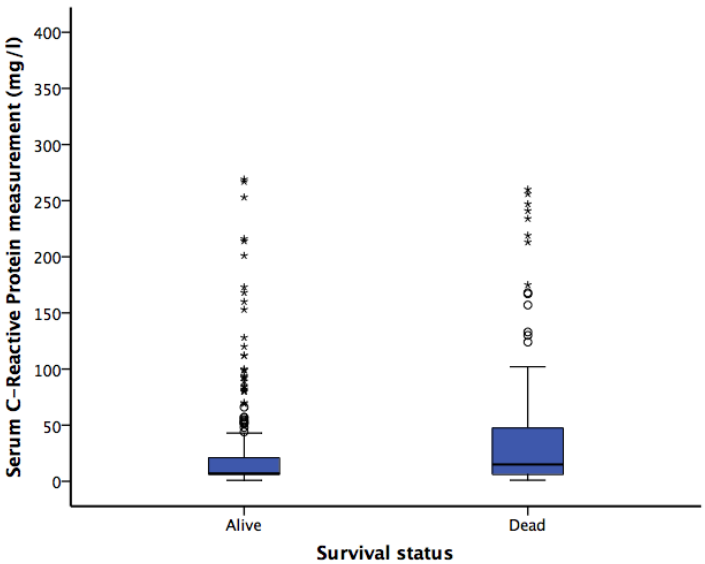
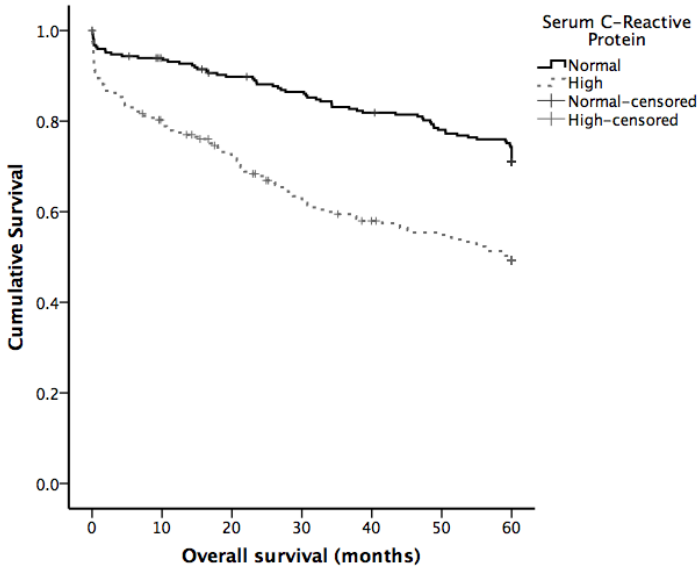


Figure 3.61: The distribution of serum CRP measurements stratified by survival status in patients with CI colorectal cancer ($p<0.001$)

The 5 year overall survival rate for patients with a raised serum CRP was 42.0% compared to 64.9% in patients with a normal serum CRP (p<0.001, Pearson's chi square). On logrank analysis raised serum CRP was significantly associated with poorer overall survival (p<0.001, figure 3.62). The mean survival for patients with a raised serum CRP was 39.7 months (95% CI 36.4 – 42.9) compared with 51.6 months (95% CI 49.4 – 53.8) in the normal serum CRP group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	248	228	216	207	196	186	177
High	219	171	150	127	114	107	97

Figure 3.62: The relationship between serum CRP expression and overall survival in patients with CI colorectal cancer (p<0.001)

Kaplan-Meier curve demonstrating the proportion of patients surviving stratified by serum CRP expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis raised serum CRP was significantly associated with poorer overall survival when dichotomised as a categorical variable (HR 2.22 (95% CI 1.64 – 3.00), p<0.001). Serum CRP was also significantly associated with poor overall survival when included as a continuous variable (HR 1.00 95% CI 1.00 – 1.01, p<0.001). The Kaplan-Meier plot suggest that serum CRP may violate the proportionality assumption of the Cox proportional hazards model. There appears to be a greater proportion of events in the high expression group near the origin of the curve. The gradient associated with these events does not appear hold throughout follow-up, suggesting that the HR is not constant and violates the proportionality assumption. However, when assessing the log(-log(survival)) plot (figure 3.63), categorical serum CRP demonstrates parallel curves and

there is no evidence of time dependency when an interaction term between serum CRP and log(time) was placed in a Cox proportional hazard model (HR 0.88 (95% CI 0.75 – 1.03); p=0.121). These findings show that serum CRP meets the proportionality assumptions of the Cox proportional hazards regression model.

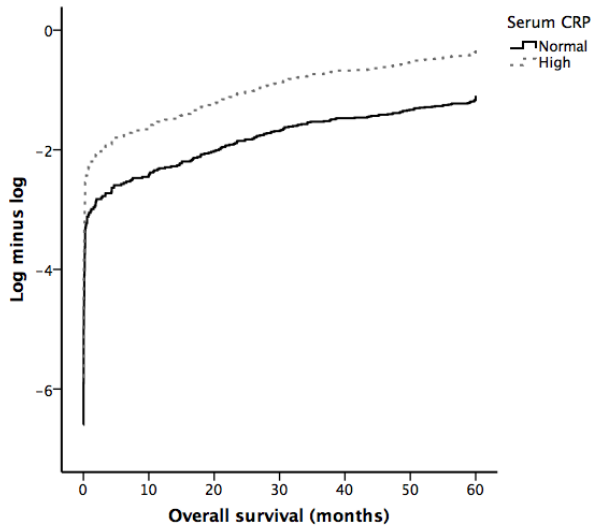


Figure 3.63: Log minus log plot of serum CRP and overall survival in patients with CI colorectal cancer

Despite the significant associations observed between serum CRP and poorer survival, its predictive value is relatively poor. When ROC analysis was performed using death as the endpoint, the AUC was 0.63 (95% CI 0.58 – 0.68, p<0.001) for serum CRP as a categorical variable with an AUC of 0.61 (95% CI 0.55 – 0.66, p<0.001) for CRP as a continuous variable (figure 3.64).

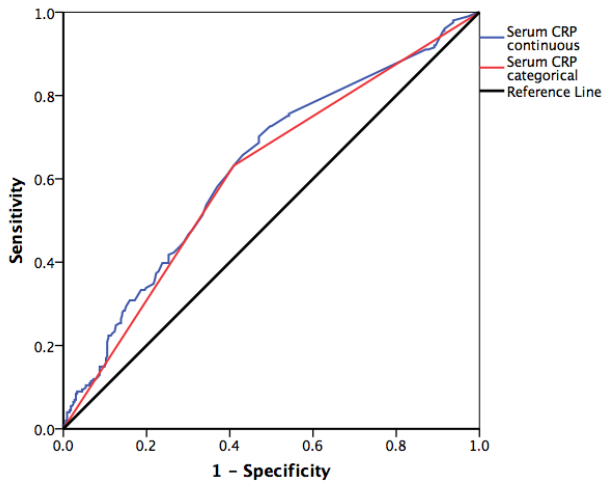


Figure 3.64: Predictive value of CRP in identifying patients with CI colorectal cancer who died during follow-up

Receiver-operator-characteristic curve demonstrating the predictive value of serum CRP in identifying patients who will die during follow-up.

Univariable overall survival - serum albumin

Serum albumin measurements were significantly lower in patients who died during the follow up period (p<0.001, Mann-Whitney U, figure 3.65). The median serum albumin was 40 g/l (IQR 33 – 47 g/l) in the alive group compared to 37 g/l (IQR 26 – 48 g/l) in patients who did not survive 5 years.

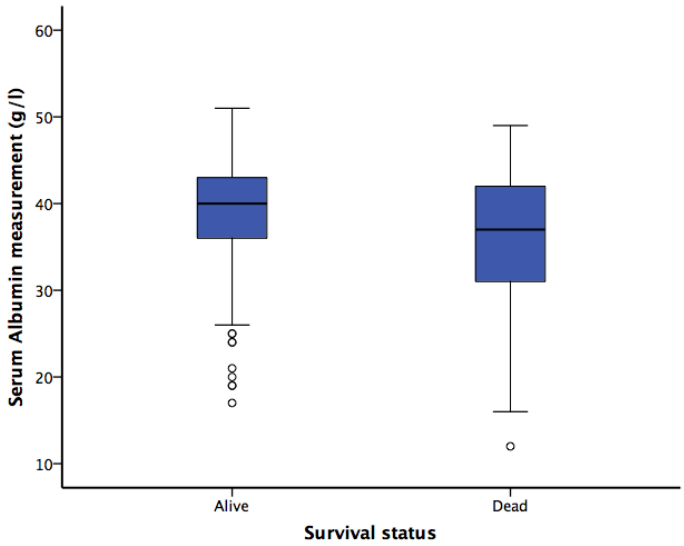
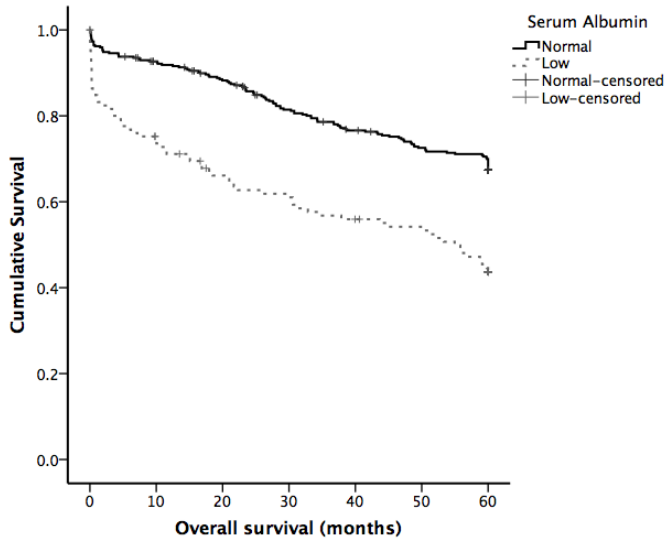


Figure 3.65: The distribution of serum albumin measurements stratified by survival status in patients with CI colorectal cancer (p<0.001)

The 5 year overall survival rate for patients with hypoalbuminaemia was 40.2% compared to 65.3% in patients with a normal serum albumin (p<0.001, Pearson's chi square). On logrank analysis hypoalbuminaemia was significantly associated with poor overall survival (p<0.001, figure 3.66). The mean survival for patients with hypoalbuminaemia was 37.4 months (95% CI 32.9 – 41.9) compared with 49.5 months (95% CI 47.6 – 51.5) in the normal serum albumin group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	369	336	316	286	267	251	242
Low	127	91	78	73	65	61	51

Figure 3.66: The relationship between serum albumin expression and overall survival in patients with CI colorectal cancer (p<0.001)

Kaplan-Meier curve demonstrating the proportion of patients surviving stratified by serum albumin expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis hypoalbuminaemia was significantly associated with poor overall survival when dichotomised as a categorical variable (HR 2.25 (95% CI 1.67 – 3.03), p<0.001). Hypoalbuminaemia was also significantly associated with poor overall survival when included as a continuous variable (HR 0.94 (95% CI 0.92 – 0.96), p<0.001). The Kaplan-Meier plot suggests that serum albumin may violate the proportionality assumption of the Cox model. There appears to be a greater proportion of events in the low expression group near the origin of the curve. The gradient associated with these events is not constant throughout follow-up, suggesting that the hazard ratio violates the proportionality assumption. When assessing the log(-log(survival)) plot (figure 3.67), categorical serum albumin demonstrates parallel curves, however, there is evidence of time dependency when an interaction term between serum albumin and log(time) was placed in a Cox proportional hazard model (HR 0.83 (95% CI 0.72 – 0.96); p=0.013) with an associated adjustment of the HR for categorical serum albumin (HR 3.30 (95% CI 2.13 – 5.12); p<0.001) (table 3.4).

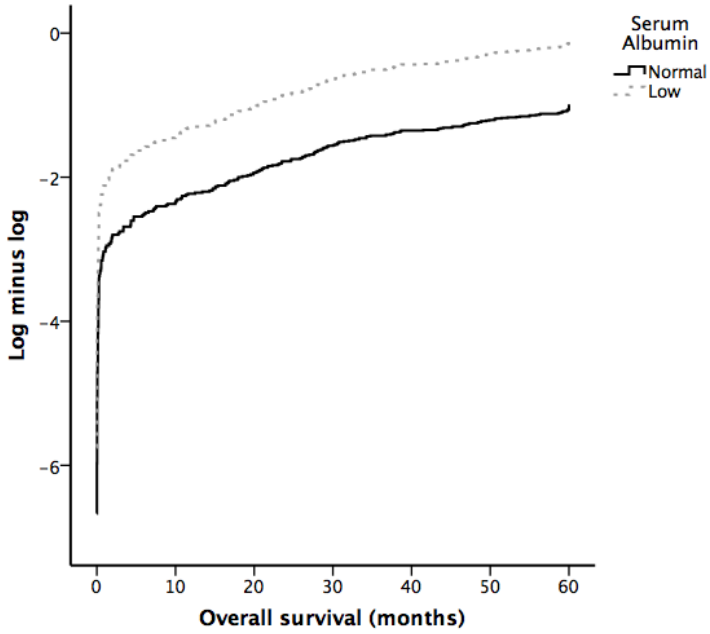


Figure 3.67: Log minus log plot of serum albumin and overall survival in patients with CI colorectal cancer

In terms of survival prediction, when ROC analysis was performed using death as the endpoint, the AUC was 0.37 (95% CI 0.32 – 0.42, p<0.001) for albumin as a continuous variable and with an AUC of 0.59 (95% CI 0.54 – 0.64, p=0.001) for albumin as a categorical variable (figure 3.68).

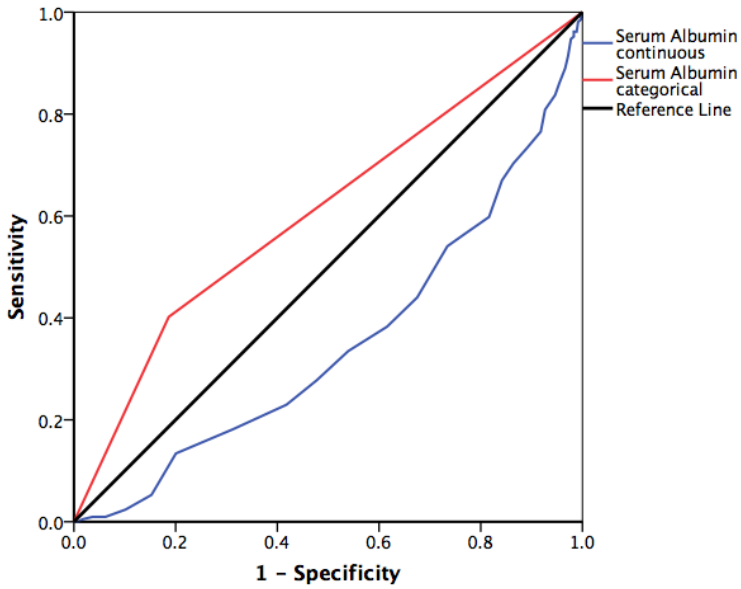


Figure 3.68: Predictive value of serum albumin in identifying patients with CI colorectal cancer who died during follow-up

Receiver-operator-characteristic curve demonstrating the predictive value of serum albumin in identifying patients who will die during follow-up.

Multivariable recurrence-free survival

Proportionality assumptions testing

All the studied variables underwent Cox proportional hazards assumption testing as described in the statistical methodology section 2.7.8.7.

Cox proportional hazards regression analysis

All covariates with a significance level of $p<0.1$ were included in the multivariable model. Terms were removed if their respective p-values were >0.05. On univariable analysis, advancing age ($p=0.048$), emergency presentation ($p<0.001$), advancing T-stage ($p<0.001$), advancing N-stage ($p<0.001$), higher TNM stage ($p<0.001$), poor differentiation (p0.002), raised serum CRP ($p=0.012$), hypoalbuminaemia ($p=0.022$) and good Klintrup score ($p<0.001$) had a p-value <0.1 and were therefore included in the multivariable Cox proportional hazards regression model.

On multivariable analysis, advancing T-stage (when all subcategories were compared with T1 (T2 HR 0.56 (95% CI 0.10 – 3.08), (T3 HR 0.97 (95% CI 0.25 – 4.35) and (T4 HR 3.06 (95% CI 0.73 – 12.87)), $p<0.001$), advancing N-stage (when all subcategories were compared with N0 (N1 HR 2.15 (95% CI 1.40 – 3.32), (N2 HR 3.02 (95% CI 1.80 – 5.07), $p<0.001$) and good Klintrup score (HR 0.52 (95% CI 0.32 – 0.84), $p=0.008$) were independently and significantly associated with poor recurrence-free survival (table 3.16).

Table 3.16: The relationships between clinicopathological factors and recurrence-free survival in patients with CI colorectal cancer: univariable and multivariable **analysis**

	Univariable analysis			Multivariable analysis		
	Coefficient	p-value	Hazard ratio (95% CI)	Coefficient	p-value	Hazard ratio (95% CI)
Age	-0.015	0.048	0.99 (0.97 - 1.00)		0.706	
Sex						
Female			1			
Male	-0.019	0.910	0.98 (0.70 – 1.37)			
Presentation						
Elective			1			
Emergency	0.645	<0.001	1.91 (1.36 – 2.68)		0.298	
Tumour site						
Right	0.000	0.131	1			
Left	-0.397		0.67 (0.45 – 0.99)			
Rectum	-0.095		0.91 (0.60 – 1.37)			
T stage						
1	0.000	<0.001	1	0.000	<0.001	1
2	-0.186		0.83 (0.17 – 4.12)	-0.574		0.56 (0.10 – 3.08)
3	0.927		2.53 (0.62 – 10.32)	0.035		0.97 (0.25 – 4.35)
4	1.893		6.64 (1.63 – 27.11)	1.120		3.06 (0.73 – 12.87)
N stage						
0	0.000	<0.001	1	0.000	<0.001	1
1	1.048		2.85 (1.96 – 4.16)	0.766		2.15 (1.40 – 3.32)
2	1.663		5.27 (3.41 – 8.15)	1.106		3.02 (1.80 – 5.07)
TNM stage						
I	0.000	<0.001	1			
II	1.088		2.97 (1.27 – 6.92)			
III	2.090		8.09 (3.53 – 18.52)		0.544	
Differentiation						
Well/Moderate			1			
Poor	0.791	0.002	2.21 (1.34 – 3.62)		0.668	
Serum CRP						
Normal			1			
High	0.484	0.012	1.62 (1.11 – 2.36)		0.576	
Serum albumin						
Normal			1			
Low	0.466	0.022	1.59 (1.07 – 2.38)		0.114	
Klintrup score						
High			1			1
Low	-1.325	<0.001	0.27 (0.16 – 0.44)	-0.842	0.008	0.52 (0.32 – 0.84)

Multivariable overall survival

Proportionality assumptions testing

All the studied variables underwent Cox proportional hazards assumption testing as described in the statistical methodology section 2.7.8.7. Age, presentation and serum albumin demonstrated time dependency when interaction terms between the covariates and log(time) were placed in the model.

Cox proportional hazards regression analysis

All covariates with a significance level of $p<0.1$ were included in the multivariable model. Terms were removed if their respective p-value was >0.05. On univariable analysis, advancing age ($p<0.001$), emergency presentation ($p<0.001$), advancing T-stage ($p<0.001$), advancing N-stage ($p=0.001$), higher TNM stage ($p<0.001$), poor differentiation ($p<0.001$), raised serum CRP ($p<0.001$), hypoalbuminaemia ($p<0.001$) and high Klintrup score ($p<0.001$) had a p-value <0.1 and were therefore included in the multivariable Cox proportional hazards regression model.

On multivariable analysis, advancing age (HR 1.05 (95% CI 1.03 – 1.08), $p<0.001$), emergency presentation (HR 2.09 (95% CI 1.31 – 3.35), $p=0.002$), T-stage (when all subcategories were compared with T1 (T2 HR 1.18 (95% CI 0.34 – 4.13), (T3 HR 1.34 (95% CI 0.42 – 4.29) and (T4 HR 2.00 (95% CI 0.61 – 6.52)), $p=0.004$), good Klintrup score (HR 0.50 (95% CI 0.33 – 0.75), $p=0.001$) and hypoalbuminaemia (HR 1.49 (95% CI 0.33 – 0.75) were independently and significantly associated with poor overall survival (table 3.17).

Influence of time dependency on outcome

Only emergency presentation ($p=0.018$) retained significant time dependency when placed in the multivariable analysis with Age ($p=0.051$) and serum albumin ($p=0.174$) becoming non-significant.

Table 3.17: The relationship between clinicopathological factors and overall survival in patients with CI colorectal cancer: univariable and multivariable analysis

	Univariable analysis			Multivariable analysis		
	Coefficient	p-value	Hazard ratio (95% CI)	Coefficient	p-value	Hazard ratio (95% CI)
Age	0.052	<0.001	1.05 (1.03 – 1.08)	0.052	<0.001	1.05 (1.03 – 1.08)
Age x log(time)	-0.009	0.012	0.99 (0.98 - 1.00)	-0.008	0.051	0.99 (0.98 – 1.00)
Sex						
Female			1			
Male	-0.059	0.657	0.94 (0.73 – 1.22)			
Presentation						
Elective			1			1
Emergency	1.135	<0.001	3.11 (2.05 – 4.73)	0.737	0.002	2.09 (1.31 – 3.35)
Presentation x log(time)	-0.175	0.014	0.84 (0.73 – 0.97)	-0.184	0.018	0.83 (0.71 – 0.97)
Tumour site						
Right	(0.000)	0.490	1			
Left	-0.180		0.84 (0.62 – 1.13)			
Rectum	-0.112		0.89 (0.64 – 1.25)			
T stage						
1	(0.000)	<0.001	1	(0.000)	0.004	1
2	-0.067		0.94 (0.38 – 2.33)	0.165		1.18 (0.34 – 4.13)
3	0.342		1.41 (0.67 – 3.20)	0.291		1.34 (0.42 – 4.29)
4	1.003		2.73 (1.23 – 6.23)	0.866		2.00 (0.61 – 6.52)
N stage						
0	(0.000)	<0.001	1		0.096	
1	0.413		1.51 (1.12 – 2.04)			
2	0.905		2.47 (1.72 – 3.55)			
TNM stage						
I	(0.000)	<0.001	1		0.225	
II	0.457		1.58 (0.99 – 2.51)			
III	0.915		2.47 (1.72 – 3.95)			
Differentiation						
Well/Moderate			1		0.114	
Poor	0.715	<0.001	2.05 (1.38 – 3.04)			
Serum CRP						
Normal			1			
High	0.796	<0.001	2.22 (1.64 – 3.00)		0.080	
Serum albumin						
Normal			1			1
Low	1.193	<0.001	3.30 (2.13 – 5.12)	0.399	0.022	1.49 (1.06– 2.10)
albumin x log(time)	-0.188	0.013	0.83 (0.72 – 0.96)		0.174	
Klintrup score						
High			1			1
Low	-0.803	<0.001	0.45 (0.32 – 0.63)	-0.694	0.001	0.50 (0.33 – 0.75)

3.4.5.9 Survival, recurrence and deaths in patients with MSI colorectal cancer

During follow-up there were 10 (11.8%) recurrences and 33 (38.3%) deaths. It has been suggested that at least 10 - 25 events are required for each variable in a multivariable model. Based on the number of events noted above, no meaningful results can be

generated by undertaking univariable and multivariable survival analysis exclusively on MSI patients. The relatively low number of patients and associated events makes the chance of incurring a type 1 or type 2 reporting error highly likely. Therefore, survival analysis has not been performed on the subset of patients with MSI colorectal cancer.

3.5 Discussion

3.5.1 Summary of the novel results

The main finding of the studies reported in this chapter is that MSI tumours are immunogenic and are strongly associated with markers of the systemic and local inflammatory responses. Furthermore, MSI tumours were associated with poor disease free survival in patients with stage III colorectal cancer.

3.5.2 Strengths and limitations

3.5.2.1 Strengths

This study has several strengths. Patient numbers in the validation cohort are relatively large by current standards and this offers an associated type II error probability of less than 0.1 when a 15% difference in 5 year survival rates were sought. In addition, all patients received stage-directed treatment by a specialist multidisciplinary team with considerable experience in treating colorectal cancer. The patients reside in a well-defined geographical area with minimal migration and full follow-up data is available for all patients.

In addition to the clinical factors associated with the patient outcome measures, measurements of the systemic inflammatory response namely serum CRP and albumin were performed by an accredited NHS biochemistry laboratory. The reliability of the serum CRP and albumin measurements also extends to the MSI analysis. Tumour MSI PCR analysis was performed in a tertiary referral laboratory and was reported under the guidance of an expert with a special interest in Lynch syndrome diagnostics.

3.5.2.2 Limitations

Data sampling and power

The main limitation of this study is the inevitable loss of statistical power when the cohorts are sub-divided eg by TNM stage and MSI status. Survival analysis was not performed in TNM stage I and MSI tumours because these groups were small in number with too few

events making the results unreliable. Although there were significant associations between biomarkers and survival in TNM stage II and stage III colorectal cancer, the multivariable model must be interpreted with caution. There was a lack of power associated with these groups and therefore, variables that may effect the strength of the covariates in the model, may not have been included because of the possibility of type II errors. Despite an adequate number of events for the number of covariates included in the model, the magnitude of hazards had a modestly significant p-value and increasing the sample size is likely to result in smaller p-values and improved power.

Another limitation of this study is the quality of the patient sample and the sample time frame. We present the frequencies of the core clinicopathological factors for the patient cohorts as well as the regional data for the study time frame. The clinicopathological frequencies in the training cohort were different to those observed in the validation cohort and the regional population. This would normally mean interpreting the pilot results with caution, however, including a validation cohort with no overlapping of patients adds some reliability to the data findings. In addition to the above, the long time frame increases the risk of heterogeneity in treatments, staging and survival.

While death is a relatively robust endpoint, however, the cause of death is dependent upon the interpretation of the clinical situation by the clinician writing the death certificate. This limitation also applies to the process of determining if cancer recurrence has developed or not. Furthermore, patients who die of an apparently unrelated illness may harbor cancer recurrence. Although CT scans can detect cancer recurrence at an early stage, a normal scan does not rule out small metastasis. Therefore, determining the exact timing of cancer recurrence and when it becomes clinically relevant is challenging.

A further limitation of this study is the non-availability of some previously reported prognostic clinicopathological factors for inclusion in the multivariable model. Although core factors such as TNM stage and its components were included, there were notable omissions such as BMI and vascular invasion. BMI is not included in the core data set for MDT discussion because of its associated limitations. Detection of venous invasion can be increased from 18% to 56% by using elastica H&E staining (Roxburgh, 2010a). This has become routine in Glasgow but only a small proportion of patients in cohorts 1 and 2 had undergone elastic H&E assessment. Therefore, with H&E assessment alone being associated with a substantial false negative rate, vascular invasion was not included in the multivariable model. BMI is a modifiable clinicopathological factor that can be considered as time dependent. Changes in living circumstances may alter a patients' BMI and thus the biological effect of this covariate on the pathophysiology of the host and the cancer varies

with time. This may explain why the effect of these factors on prognosis remains poorly understood. A metanalysis of 58917 patients showed that pre- and post-diagnosis low BMI was associated with poor cancer-specific survival. However, post-diagnosis obesity was not associated with cancer-specific survival whilst being overweight inferred a significant but modest improvement in cancer-specific survival (Lee, 2015). The reliability of these observations in cohorts of prospective studies makes translating these variables into firm prognostic markers challenging.

A minor limitation of this study is that tumours were dissected macroscopically rather than microscopically to increase the percentage of tumour DNA in the sample. It has been estimated that a sample requires at least 40% tumour DNA to detect the microsatellite repeats reliably on PCR. Factors that potentially dilute the tumour DNA include lower T stage tumours and a pronounced inflammatory infiltrate. These features increase the volume of non-cancerous DNA thus increasing the false negative detection rate. MSI tumours, which are associated with a pronounced inflammatory infiltrate and lower T-stage tumours, have a higher proportion of adjacent adenoma and thus require dissection (Soreide, 2006). Macroscopic dissection may inadvertently include a proportion of these non-cancerous cells in the sample thus diluting the tumour DNA yield. To control for this, all tumours underwent MMR protein assessment through immunohistochemistry; however, an MMR deficient/MSI negative phenotype was observed in 4.7% of cases and it is unclear if this represents a spurious result of immunohistochemistry or PCR.

3.5.3 Discussion of the results

3.5.3.1 MSI tumours and the systemic inflammatory response

The results presented in this chapter demonstrate an association between the SIR and MSI colorectal cancer. The causation of this relationship is unclear and cannot be explained by a purely observational study. Several possible explanations exist for the association of the SIR and colorectal cancer. Firstly, the SIR may be a *consequence* of the tumour related complications (eg tumour perforation) or caused by the primary tumour or metastatic tumour load. Secondly, a pre-existing SIR may contribute towards the development of colorectal cancer in particular, MSI tumours.

In cardiovascular disease and diabetes, poor diet, obesity and smoking were closely related to an elevated CRP and poorer prognosis (Sattar, 2003). Modification of these lifestyle factors resulted in the resolution of the SIR. This supports the concept of the systemic inflammatory response being a time-dependent modifiable prognostic marker.

There is evidence that poor diet, obesity and smoking are associated with the development of MSI and MMR-D colorectal cancers (Limsui 2010, Samowitz 2006, Slattery 2006). There are at least two mechanisms for the development of MSI tumours, firstly germ-line mutations in the MMR genes (as in the hereditary cancer syndrome, HNPCC; Kambara, 2004) and secondly, silencing of the MLH1 gene by hypermethylation of its promoter region in association with the CpG Island Methylator Phenotype (CIMP) results in sporadic colorectal cancer (Toyota, 1999). Adverse lifestyle features associated with the metabolic syndrome have also been associated with DNA hypermethylation (Barres, 2011). Two patterns of DNA methylation have been proposed, cancer-specific and whole genome non-specific methylation (Toyota, 1999). While there are associations between adverse lifestyle factors, metabolic syndrome, the SIR, DNA methylation and MSI colorectal cancer, mechanistic studies are still required to uncover causal relationships.

3.5.3.2 MSI and survival

In the patient cohorts reported in this chapter, MSI was not independently associated with improved recurrence free survival. MSI is widely accepted as a biomarker of relatively improved outcome when compared with CI (MSS) colorectal cancer. A systematic review and meta-analysis of 7,642 patients found that the HR for MSI was 0.65 (95% CI 0.59 – 0.71) for overall survival when compared to CI (Popat, 2005). This observed hazard ratio, however, was not adjusted for confounding factors. Indeed, the hazard ratios observed for MSI and recurrence-free survival in the whole validation cohort and TNM stage II were 0.50 and 0.42 respectively; however, following multivariable survival analysis MSI status was not independently associated with survival. This suggests that the prognostic power of MSI is based on its associations with lower tumour stage and a pronounced local inflammatory response and these retain independence in the constructed models. MSI does not appear to be prognostically superior to other factors; however, in terms of treatment stratification predictive biomarkers could have greater clinical importance. MSI tumours are relatively chemoresistant to 5-FU and do not respond to EGF receptor inhibitors, possibly due to BRAF mutations, so MSI may remain an important biomarker for treatment stratification.

3.5.3.3 SIR in predicting survival

The association of the SIR and survival in colorectal cancer has been widely studied. Several methods for quantifying the SIR have been proposed based on absolute measurements of the full blood count components (White cell count, Neutrophil count,

Lymphocyte count and Platelet count), their derivatives (NLR and PLR) and the protein markers CRP, albumin and mGPS (Roxburgh, 2010b). In the studies reported here CRP and albumin were used as markers of the SIR. Both NLR (Shibutani, 2015; Chen, 2015) and PLR (Zou, 2016) are reported as independent predictors of poor outcome Roxburgh *et al* found that only the CRP and albumin based mGPS was independently associated with poor outcome (Roxburgh, 2010b). Although initial evidence suggests that CRP, albumin and the mGPS offer superior prognostic information over other methods of SIR quantification, independent validation in an appropriately powered cohort is required.

Low serum albumin was independently associated with poor overall survival in the entire cohort as well as in the TNM stage II group. This is similar to the findings of Heys *et al* (Heys, 1998), Sun *et al* (Sun, 2009) and Ishizuka *et al* (Ishizuka, 2007). The underlying mechanisms are unclear. More aggressive tumours could inherently stimulate more inflammation but equally systemic inflammation could promote more aggressive behaviours by a tumour. Lai *et al* observed in 3,849 patients that hypoalbuminaemia was associated with larger tumours, T4 disease, poor differentiation as well as cardiovascular disease and diabetes (Lai, 2011). In clinical practice, the SIR can be described as acute or chronic and both are commonly seen in the emergency and elective settings respectively. It is possible therefore that both the acute and chronic SIRs have different causes and effects on the pathophysiology of the cancer.

It has been discussed previously that the SIR could promote contribute to the formation of MSI colorectal cancer, however, there is also evidence that the SIR contributes to the malignant potential of the cell through activation of signaling pathways. Canna *et al* observed a correlation between serum CRP levels and tumour cell proliferation as measured by Ki67 immunohistochemistry (Canna, 2008). This was an observational study, the finding that Il-6 drives cell proliferation via the JAK/STAT3 pathway in colitis-associated colorectal cancer in mice supports the concept of an interaction between the SIR and cancer cell behaviour (Grivennikov, 2009). Grivennikov *et al* observed lower proliferative activity, (measured by nuclear Ki67), in Il-$6^{-/-}$ tumours compared to Il-6 wild type. Furthermore, ablation of STAT3 in cultured normal colonic epithelial cells resulted in reduced tumour growth. However, it remains unknown if this will translate into in-vivo human colorectal cancers.

Tumour growth is based on a balance between increased cancer cell proliferation, loss of apoptosis and loss of the cytotoxic effects of the immune response. In addition to colorectal cancer cell proliferation, activation of STAT3 has also been implicated in regulating apoptosis (Lin, 2005; Xiong, 2008), which may partly explain why the SIR is

associated with larger tumours (Crozier, 2007). Xiong *et al* observed that inhibition of the JAK1, 2/STAT3 pathway induced colorectal cancer cell apoptosis (Xiong, 2008). They inhibited the pathway using siRNA for STAT3 inhibition and the JAK inhibitor, AG490. Inhibition of Il-6 and its effects on apoptosis has not been studied in colorectal cancer. However, neutralising endogenous Il-6 with an anti-Il-6 antibody reduced the survival of Doxorubicin-treated hepatocellular cancer cells probably by restoring apoptosis (Liu, 2010). In vivo, JAK/STAT signalling is likely to come from a number of stimuli and further studies of the role of Il-6 inhibition on JAK/STAT3 mediated apoptosis in colorectal cancer are required.

Personalised cancer medicine aims to improve the efficacy of treatments through identifying patients most likely to benefit from particular treatments. Despite the identification of many candidate biomarkers, few have made it into clinical practice. Prognostic biomarkers add little beyond TNM stage in the management of colorectal cancer. The majority of biomarkers that have been successfully introduced into clinical practice are predictive in nature as they help guide treatment through patient identification. Spontaneous apoptosis has been associated with improved response to neoadjuvant chemotherapy in rectal cancer (Rödel, 2002). The results by Liu *et al* described above suggest that the SIR may act as a predictive biomarker for poor response to chemotherapy through its suppression of cellular apoptosis. This may explain the findings by Carruthers *et al* that the SIR was associated with poor survival in patients undergoing neoadjuvant therapy for rectal cancer (Carruthers, 2012). Furthermore, a raised NLR conferred a poorer outcome in patients receiving neoadjuvant gemcitabine +/- nab-paclitaxel in metastatic pancreatic cancer (Goldstein, 2015). Factors that influence overall survival are multifactorial and these observations may not be tumour-specific. Therefore, confirmatory evidence examining the association of the SIR and pathological response to neoadjuvant therapy is required to establish the predictive value of these biomarkers and their role in clinical practice.

3.5.3.4 Introducing MSI status and the SIR into the prognostication process in colorectal cancer

Following potentially curative resection and histopathological assessment of the specimen, patients are offered adjuvant chemotherapy if there are features suggestive of higher risk of disease recurrence. These currently include T4 disease, lymph node metastasis and vascular invasion. As discussed previously, TNM staging remains the main biomarker for

outcome prognostication; but its predictive value could be better. Methods to strengthen the predictive value of TNM stage with supplementary biomarkers would improve allocation patients to adjuvant treatment on a need/benefit basis. Supplementary options could include MSI status and inflammatory markers.

The distinction between staging, prognosis and predictive biomarkers is important given the influence of both on survival and treatment. TNM staging does a reasonable prognostic job but has little value in predicting response to different adjuvant therapies. Both breast and oesophageal cancer have tumour grade incorporated into the 7th edition of TNM staging system, however anecdotally, minimal benefit has been observed by including this supplementary information. Grading or differentiation is notoriously difficult to accurately establish with poor inter-observer reproducibility and therefore different biomarkers are needed (Chandler, 2008). In breast cancer, ER, PR and HER2 status offer useful information for treatment stratification with biological therapies. It appears that the main barrier for implementing biomarkers into clinical practice is reliable biomarker identification, quantification and reporting, and availability of a specific therapy with a favorable benefit/side effect profile. There is a clear need for treatment stratifying biomarkers in colorectal cancer, however, it remains unclear how both the SIR and tumour MSI will be of use in this setting.

MSI tumours are chemoresistent to 5-FU and MSI is therefore predictive of non-response to adjuvant chemotherapy regimens containing it or Capecitabine. Given the relatively improved survival observed in MSI tumours it is possible that some patients with these tumours should not be offered adjuvant chemotherapy at all, but there will be a subgroup of MSI tumours, eg stage III, likely to have a poor outcome and delineating the pathophysiological cause for non-response offers a novel therapeutic target. The reason for the poor response of MSI tumours to chemotherapy is unclear, however, the role of the SIR, through Il-6 and STAT3, requires further study. Anti-inflammatory agents may sensitise these tumours to chemoradiotherapy; this requires exploration. Although MSI tumours are associated with the SIR they have a relatively improved outcome, possibly due to the infiltration of cytotoxic CD8+ lymphocytes, and therefore not all patients will require adjuvant therapy. Stage I and II MSI tumours could be offered surveillance only, thus avoiding adjuvant chemotherapy and its associated side effects.

The case for utilising MSI as a predictive biomarker for adjuvant treatment is rather compelling, however, the role of the SIR is still in the early stages. The evidence here demonstrates that the SIR is strongly associated with poor survival and is independent of other commonly used clinicopathological factors. However, until the relationship between

the SIR and pathological response to chemoradiotherapy has been established, it is unlikely that markers of the SIR will be integrated into the TNM staging system or guide adjuvant treatment. Delineating the pathophysiological interaction between the tumour and the host may establish the SIR as a novel therapeutic target for high risk patients.

3.5.4 Future direction

1. Given the lack of power associated with studying survival associations in each TNM stage and MSI tumours, future work will need to validate these findings in a large sample size, which is adequately powered.

2. How the SIR influences the development of MSI colorectal cancer is unclear, however, there is a possible link with DNA methylation. Future work examining the association between the SIR, DNA methylation, CIMP and MSI colorectal cancer which is supported by mechanistic experiments could identify therapeutic targets for MSI colorectal cancer prevention.

3. The biological reasons for the association between the SIR and survival remains unknown. Future work examining the relationship between the SIR and cell signaling pathways such as JAK/STAT3 may help identify novel therapeutic targets.

3.5.5 Conclusion

The SIR is significantly associated with poorer outcome in colorectal cancer and incorporating this biomarker into staging may help identify patients who require adjuvant treatment. Given the association between the SIR with MSI colorectal cancer the causes of this relationship needs to be identified, as not all patients will require more aggressive treatment.

4. The role of Matrix Metalloproteinase 9 (MMP-9) in colorectal cancer progression and survival

4.1 Introduction

Disease progression and metastasis depend on complex relationships between tumour behaviour and the protective influence of the local inflammatory response (Klintrup, 2005). Tumour cell invasion and metastasis is a cardinal feature of cancer and is what ultimately leads to organ dysfunction and patient death.

The mechanisms by which colorectal cancer cells locally invade through the bowel remain uncertain, but connective tissue degradation by matrix metalloproteinases such as MMP-9 have been implicated. Lubbe *et al* (2006) found high levels of MMP-9 mRNA in stroma as well as in epithelial cancer cells. Furthermore, MMP-9 in colorectal tissue was associated with degradation of extracellular matrix components (Lubbe, 2006) and poor survival. Although at one time it was thought that MMP-9 in colorectal cancer was secreted by macrophages, MMP-9 mRNA has been identified in both cancer cells and stromal cells, with MMP-9 protein also being observed in the circulation. MMP-9 in the serum was not diagnostic of colorectal cancer in a screening study and is therefore not wholly a result of tumour production. What influence, if any, serum MMP-9 has on the tumour remains unknown.

The hypotheses to be tested in this study were two fold. Firstly, that MMP-9 will be associated with CI colorectal cancer and poor outcome, independent of commonly used clinicopathological factors. Secondly, that serum MMP-9 will be associated with tumour expression and poor outcome.

4.2 Training cohort (cohort 1)

4.2.1 Colorectal cancer MMP-9 expression

Cancer cell MMP-9 expression was evaluated using immunohistochemistry and the weighted histoscore as previously described in section 2.6.61. Tumour cell membrane and nuclear immunoreactivity were not observed in any tumours while 100.0% of tumours demonstrated at least some cytoplasmic immunoreactivity (figure 4.1).

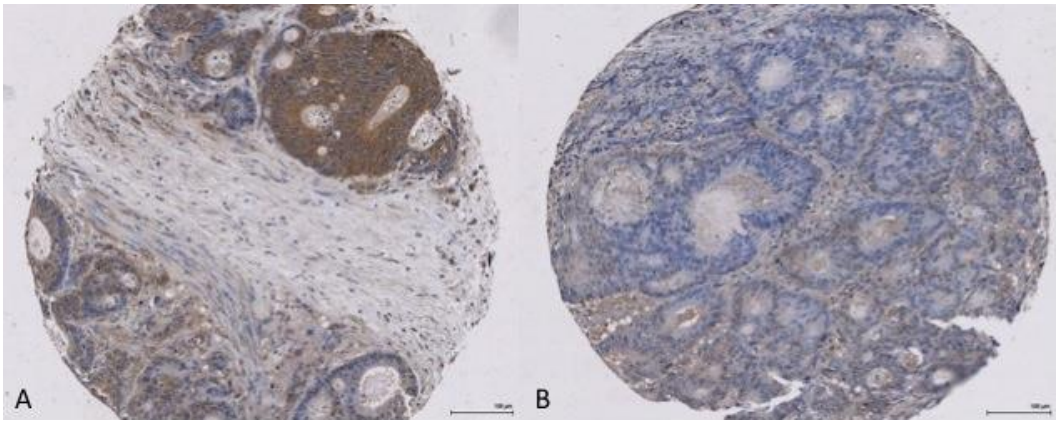
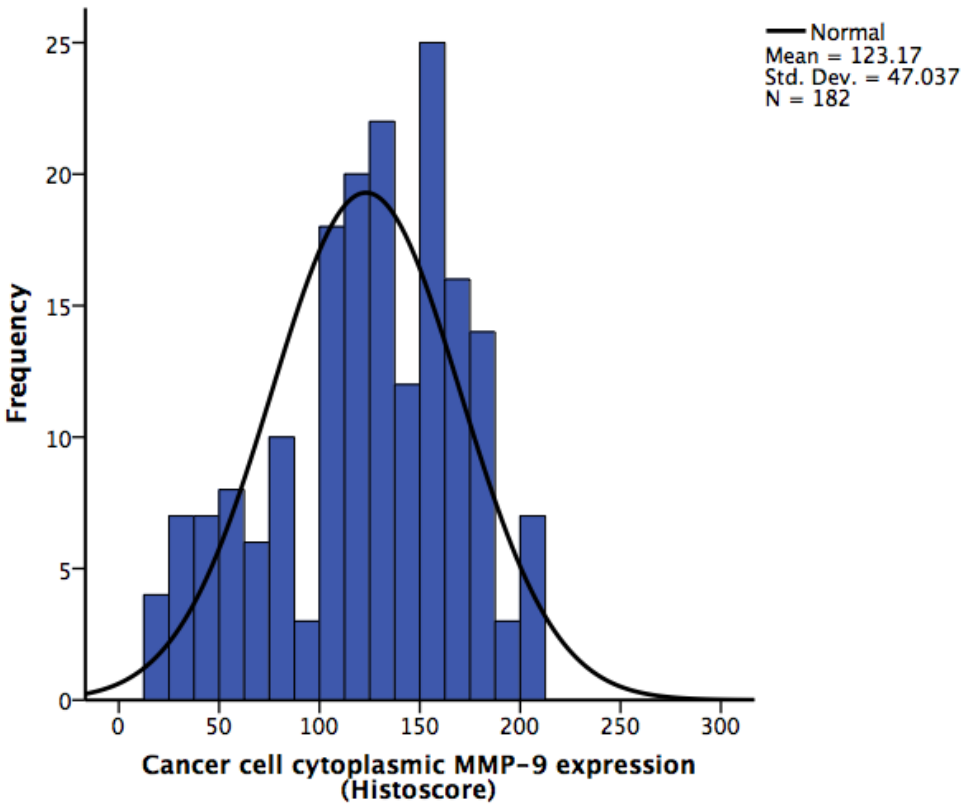


Figure 4.1: MMP-9 Immunohistochemistry

Representative areas of cancer cells showing high and low cytoplasmic MMP-9 expression (A and B)

4.2.1.1 Distribution of cancer cell cytoplasmic MMP-9 expression

Figure 4.2 demonstrates that the expression of tumour cell cytoplasmic MMP-9 does not precisely follow a normal distribution (histogram, figure 4.2), which is supported by a Shapiro-Wilk statistic of 0.961, df 182, p<0.001. The measurements ranged from 15.00 – 206.33 with a median of 127.09 (IQR 65.84 – 188.34) (supplementary figure 4.1, appendix 5.2).



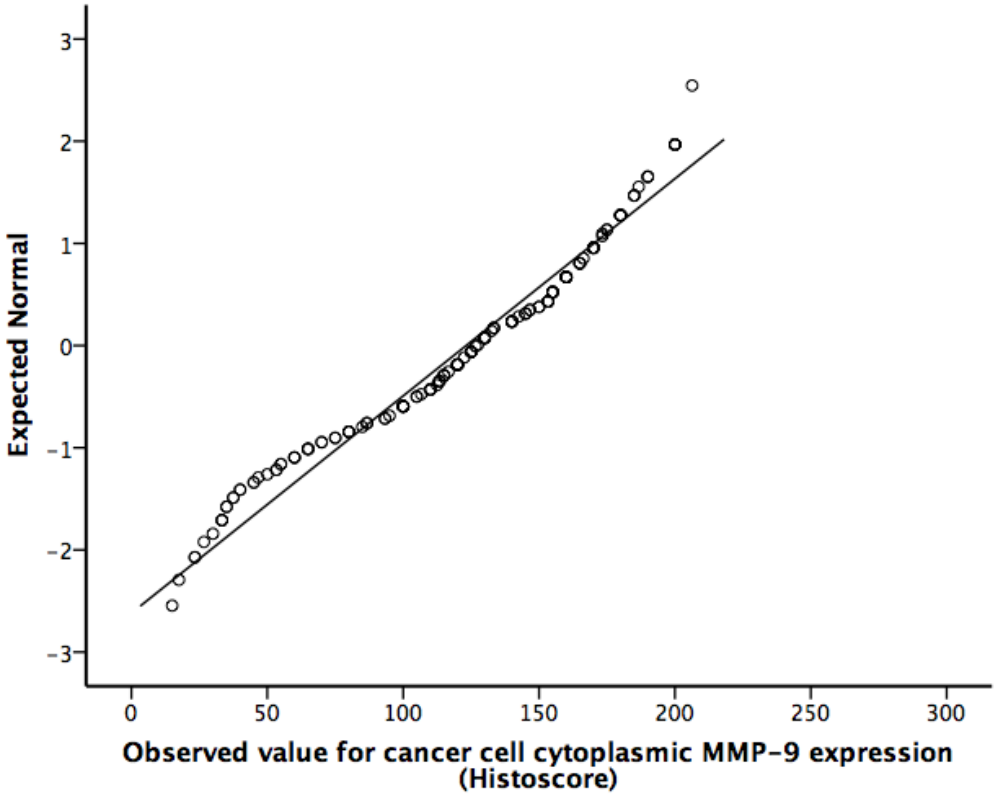


Figure 4.2: Distribution of measurements for cancer cell cytoplasmic MMP-9

Histogram demonstrating the distribution of cancer cell cytoplasmic MMP-9. Q-Q Plot of cancer cell cytoplasmic MMP-9 measurements.

4.2.2 Generation of cut offs and association with survival

Many factors influence overall survival. Tumour related biomarkers are cancer specific and therefore recurrence and recurrence-free survival have been used to choose optimal cutoffs for dichotomisation.

4.2.2.1 Tumour cancer cell cytoplasmic MMP-9 expression

When ROC analysis was performed using recurrence as the endpoint, the AUC was calculated for cytoplasmic MMP-9 as a continuous variable, and then categorised as a dichotomy around the median, tertiles and quartiles. The AUCs were 0.71 (95% CI 0.63 – 0.80, p<0.001) for cytoplasmic MMP-9 as a continuous variable, 0.67 (95% CI 0.58 – 0.76, p=0.001) for cytoplasmic MMP-9 as a median, 0.70 (95% CI 0.61 – 0.79, p<0.001) for cytoplasmic MMP-9 as tertiles and an AUC of 0.71 (95% CI 0.62 – 0.80, p<0.001) for cytoplasmic MMP-9 as quartiles (figure 4.3).

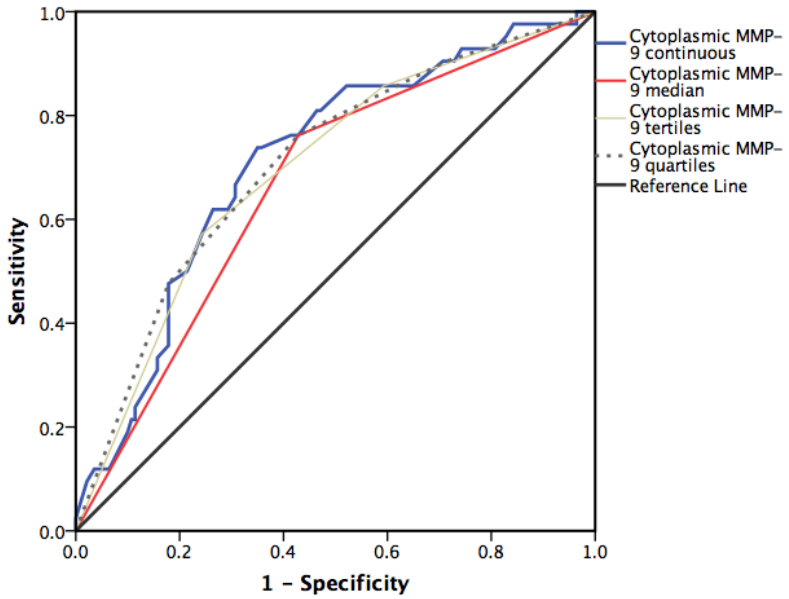
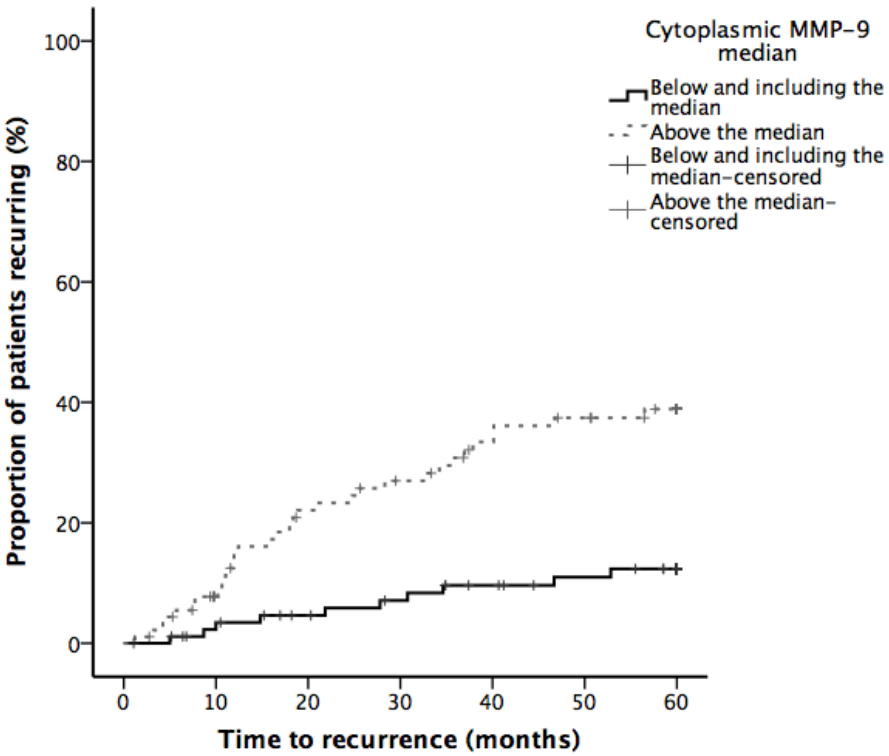


Figure 4.3: The predictive value of cytoplasmic MMP-9 in identifying patients who will develop cancer recurrence

Receiver-operator-characteristic curve demonstrating the predictive value of cytoplasmic MMP-9 in identifying patients who will develop cancer recurrence.

On Kaplan-Meier analysis, there was a significant association between cytoplasm MMP-9 expression and recurrence free survival when categorised around the median ($p<0.001$), as tertiles ($p<0.001$) and quartiles ($p<0.001$) (figure 4.4).



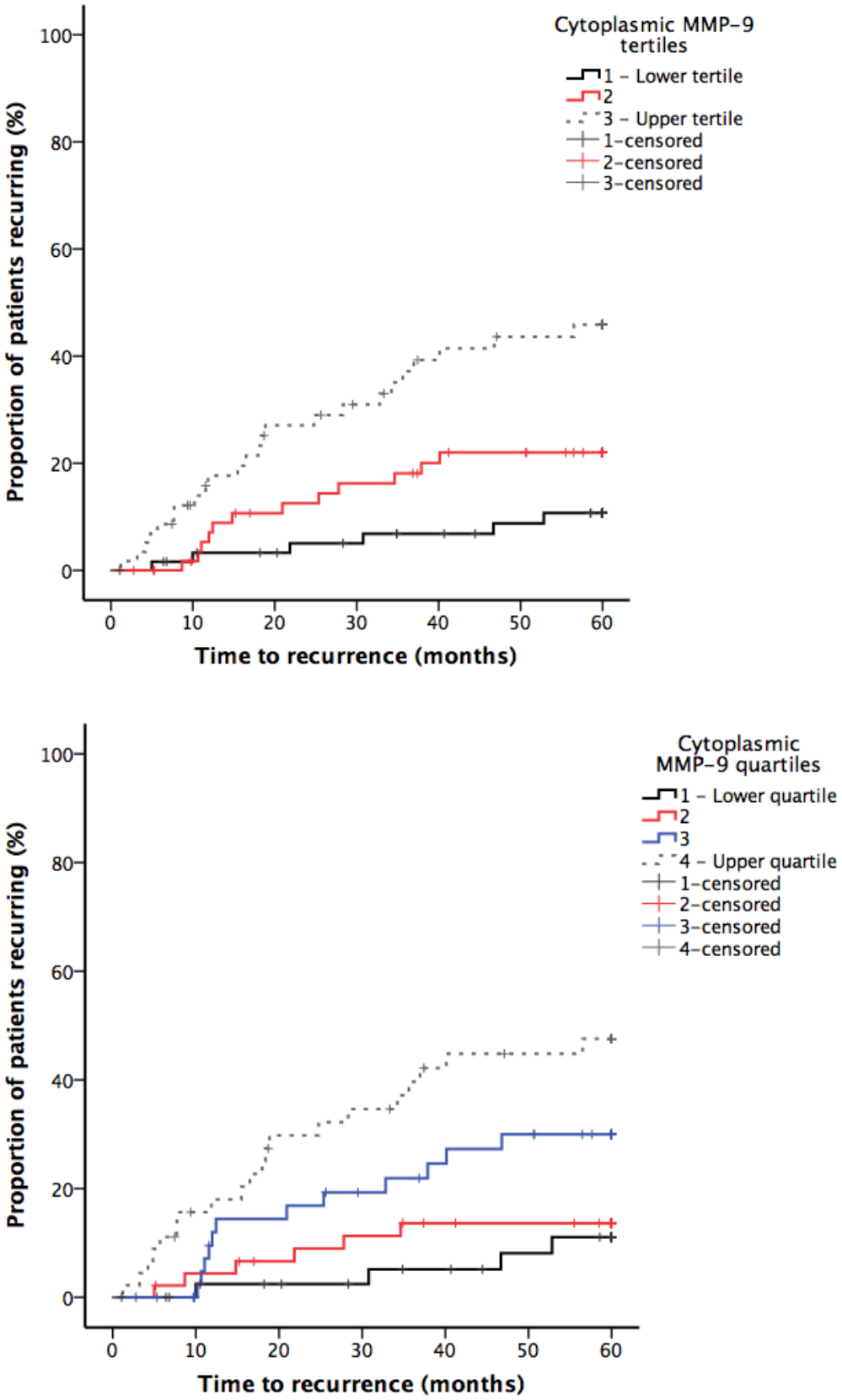


Figure 4.4: Kaplan-Meier curves demonstrating the association between cytoplasmic MMP-9 expression and recurrence-free survival in patients with colorectal cancer

4.2.2.2 Justification for choice of cutoff

The Kaplan-Meier curves demonstrate that higher expressions of cytoplasmic MMP-9 are associated with poor recurrence free survival. ROC analysis demonstrates that dichotomisation around the median has the lowest AUC when compared with the other categorical derivatives. Identifying and validating a threshold for dichotomising cytoplasmic MMP-9 may help with its incorporation into clinical practice. The ideal categorical biomarker will have a strong association with good outcome in the low group and a strong association with poor outcome in the high group or visa versa. The Kaplan-Meier of the quartile categorisation of cytoplasmic MMP-9 demonstrates that dichotomising around the upper quartile would produce a low expression groupmarkedly heterogeneous for recurrence-free survival, and therefore dichotomisation around the median has been chosen.

4.3 Validation cohort (cohort 2)

4.3.1 Colorectal cancer MMP-9 expression

Tumour MMP-9 was evaluated using immunohistochemistry and the weighted Histoscore as described in section 2.6.6.1. No tumours demonstrated MMP-9 expression at the cell membrane or nucleus, however, 91.3% of tumours demonstrated MMP-9 expression in the cytoplasm.

4.3.1.1 Cytoplasmic MMP-9

Cytoplasmic MMP-9 measurements did not demonstrate any particular distribution (figure 4.5). The measurements ranged from 0.00 – 200.00 with median of 37.50 (IQR 0.00 – 82.50) (supplementary figure 4.2, appendix 4.2). Using the dichotomisation process described in section 4.2.2.2, 49.3% of patients were considered high expressers (above the median).

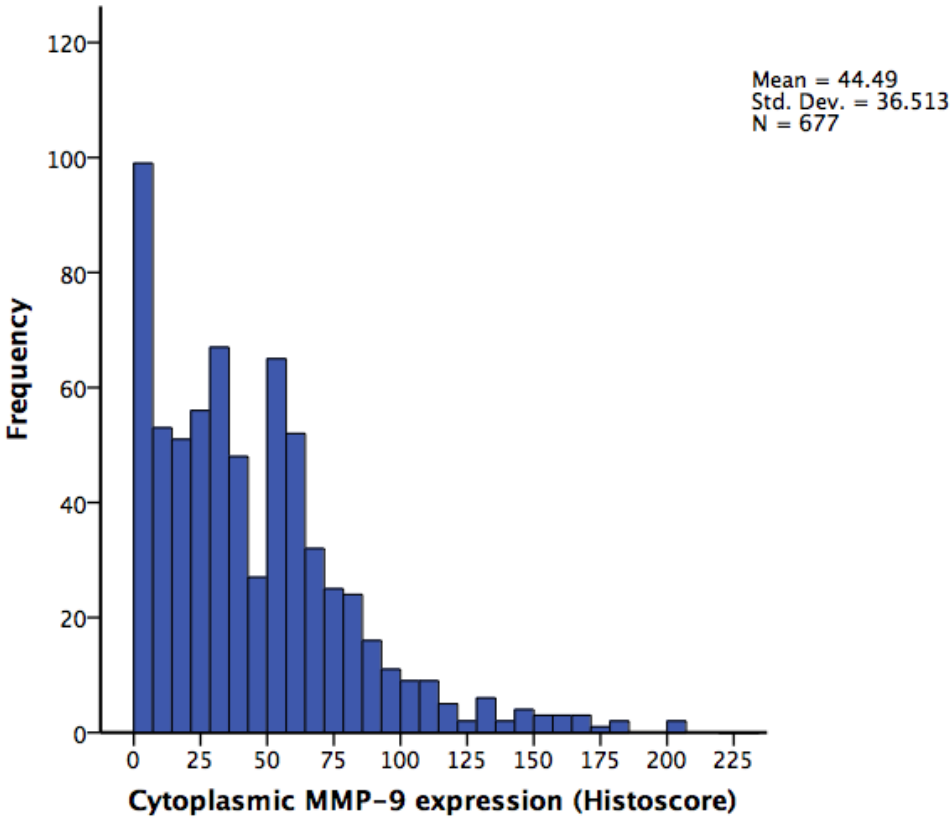


Figure 4.5: Distribution of measurements of cytoplasmic MMP-9 in patients with colorectal cancer

4.3.2 Cytoplasmic MMP-9 association with clinicopathological factors

4.3.2.1 Assessment of cytoplasmic MMP-9 as a continuous variable across different groups of categorical clinicopathological factors

Only female gender (p=0.018, Mann-Whitney U) and emergency presentation (p=0.035, Mann-Whitney U) showed a trend towards an association with higher cytoplasmic MMP-9 expression, however, these were not significant following Bonferroni adjustment (p<0.0045). Tumour site (p=0.423, Kruskal-Wallis), T-stage (p=0.265, Mann-Whitney U), N-stage (p=0.585, Kruskal-Wallis), tumour differentiation (p=0.992, Mann-Whitney U), categorical serum CRP (p=0.197, Mann-Whitney U), categorical serum albumin (p=0.310, Mann-Whitney U) and Klintrup score (p=0.762, Kruskal-Wallis) were not associated with continuous cytoplasmic MMP-9 expression.

4.3.2.2 Assessment of cytoplasmic MMP-9 as a continuous variable across different continuous data types of clinicopathological factors

On Spearman's rank test cytoplasmic MMP-9 was not associated with Age (SCC 0.043, p=0.268), serum CRP (SCC 0.095, p=0.028) or serum albumin (SCC 0.003, p=0.939).

4.3.2.3 Assessment of cytoplasmic MMP-9 associations with clinicopathological factors: categorical data type

Raised cytoplasmic MMP-9 was not associated with any of the clinicopathological factors studied. Gender (p=0.176), presentation (p=0.250), tumour site (colon vs rectum) (p=0.392), tumour site (right vs left vs rectum) (p=0.444), tumour differentiation (p=0.884), T-stage (p=0.527), N-stage (p=0.615), TNM stage (p=0.357), serum CRP (p=0.275), serum albumin (p=0.297), Klintrup score (p=0.287) and MSI status (p=0.653) were not associated with categorical cytoplasmic MMP-9 expression (table 4.1).

Table 4.1: The relationships between tumour cytoplasmic MMP-9 expression and clinicopathological factors

Clinicopathological factors	Low cytoplasmic MMP-9 expression	High cytoplasmic MMP-9 expression	p-value
Sex			
Female	177 (51.6%)	155 (46.4%)	0.176
Male	166 (48.4%)	179 (53.6%)	
Presentation			
Elective	248 (72.3%)	228 (68.3%)	0.250
Emergency	95 (27.7%)	106 (31.7%)	
Tumour Site			
Colon	266 (77.6%)	268 (80.2%)	0.392
Rectum	77 (22.4%)	66 (19.8%)	
Tumour Site			
Right	155 (45.2%)	147 (44.0%)	0.444
Left	111 (32.4%)	122 (36.5%)	
Rectum	77 (22.4%)	65 (19.5%)	
Differentiation			
Well-Mod	309 (90.1%)	302 (90.4%)	0.884
Poor	34 (9.9%)	32 (9.6%)	
T stage			
1	16 (4.7%)	11 (3.3%)	0.527
2	51 (14.9%)	40 (12.0%)	
3	178 (51.9%)	183 (54.8%)	
4	98 (28.6%)	100 (29.9%)	
N stage			
0	220 (64.1%)	219 (65.6%)	0.615
1	89 (25.9%)	77 (23.1%)	
2	34 (9.9%)	11.4%)	
TNM stage (simplified)			
I	59 (17.2%)	46 (13.8%)	0.357
II	158 (46.1%)	169 (50.6%)	
III	126 (36.7%)	119 (35.6%)	
Serum CRP			
Normal	138 (53.1%)	132 (48.4%)	0.275
High	122 (46.9%)	141 (51.6%)	
Serum albumin			
Normal	205 (75.4%)	208 (71.5%)	0.297
Low	67 (24.6%)	83 (28.5%)	
Klintrup score			
Good	245 (71.4%)	226 (67.7%)	0.287
Poor	98 (28.6%)	108 (32.3%)	
MSI status			
CI	298 (86.9%)	294 (88.0%)	0.653
MSI	45 (13.1%)	40 (12.0%)	

Bonferroni adjustment = <0.0032

* Signifies the use of Fishers exact test

4.3.3 Association of cytoplasmic MMP-9 expression, clinicopathological factors and survival – entire cohort

During follow-up there were 150 (22.2%) recurrences and 260 (38.4%) deaths. Five year recurrence-free and overall survival rates for the clinicopathological factors studied can be found in appendix 3.2.

4.3.3.1 Univariable recurrence-free survival - cytoplasmic MMP-9 expression

Tumour cytoplasmic MMP-9 measurements were not significantly different in patients who went on to develop disease recurrence (p=0.510, Mann-Whitney U, figure 4.6). The median measurement for patients with recurrence was 37.50 (IQR 0.00 – 80.63) compared with 37.50 (IQR 0.00 – 83.33) in the non-recurrence group.

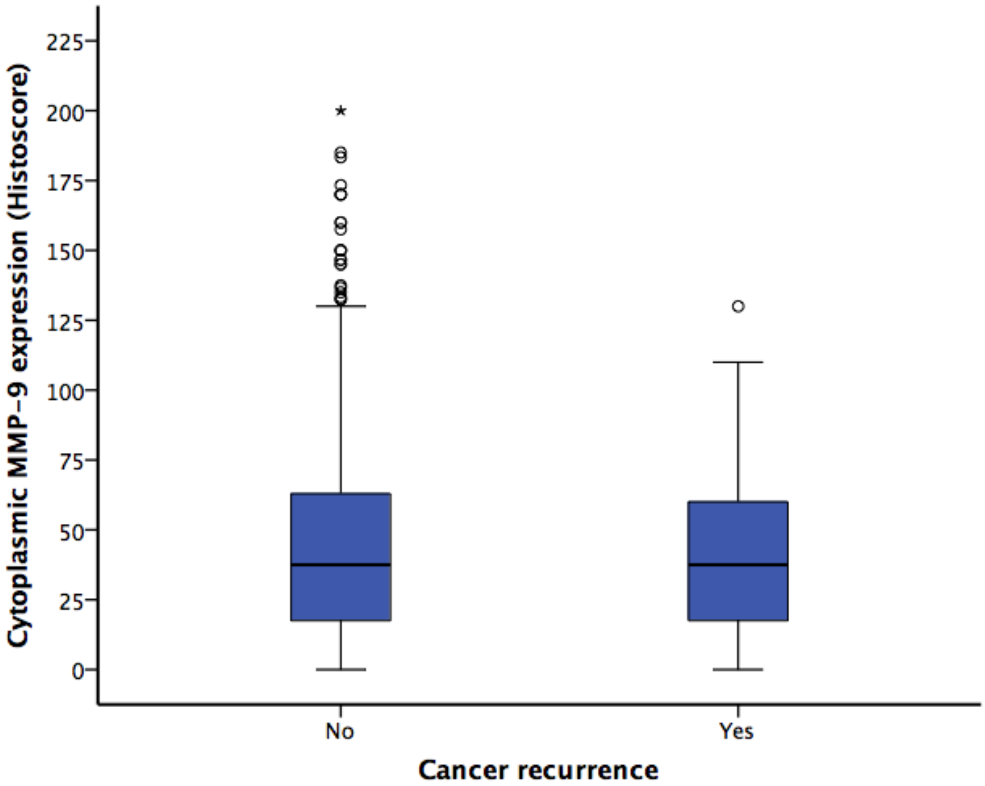
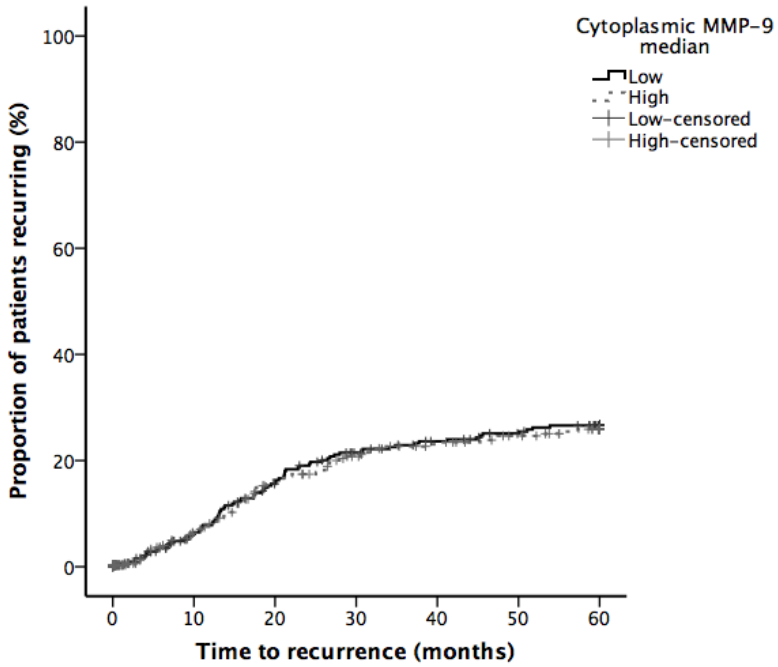


Figure 4.6: The distribution of Cytoplasmic MMP-9 measurements in patients with and without disease recurrence (p=0.510)

The 5 year recurrence free survival rate for patients with a raised cytoplasmic MMP-9 was 51.8% compared to 53.5% in patients with a low cytoplasmic MMP-9 (p=0.752, Pearson's chi square). On logrank analysis, raised cytoplasmic MMP-9 was not significantly associated with poor recurrence free survival (p=0.846, figure 4.7). The mean survival for patients with a raised cytoplasmic MMP-9 was 49.6 months (95% CI 47.4 – 51.8)

compared with 49.3 months (95% CI 47.1 – 51.4) in the low cytoplasmic MMP-9 group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	343	279	246	222	209	200	189
High	334	269	230	211	196	186	173

Figure 4.7: The relationship between cytoplasmic MMP-9 expression and recurrence-free survival in patients with colorectal cancer (p=0.846)

Kaplan-Meier curve demonstrating the proportion of patients recurring stratified by cytoplasmic MMP-9 *expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.*

On Cox univariable regression analysis raised cytoplasmic MMP-9 was not significantly associated with recurrence-free survival when dichotomised as a categorical variable (HR 0.97 (95% CI 0.70 – 1.33), p=0.846) or as a continuous variable (HR 1.00 (95% CI 0.99 – 1.00), p=0.281). In addition to the non-significant associations observed between cytoplasmic MMP-9 expression and disease recurrence, its predictive value was also non-significant. When ROC analysis was performed using recurrence as the endpoint, the AUC was 0.48 (95% CI 0.43 – 0.53, p=0.510) for cytoplasmic MMP-9 as a continuous variable and an AUC of 0.49 (95% CI 0.44 – 0.54, p=0.748) for cytoplasmic MMP-9 as a categorical variable (figure 4.8).

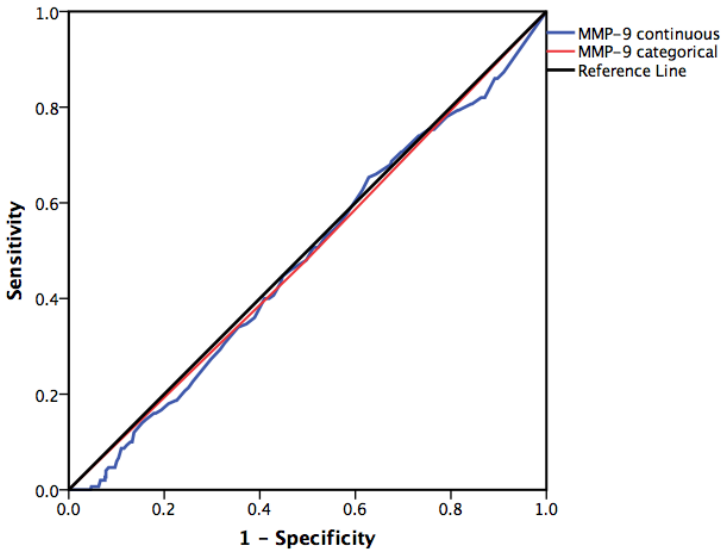


Figure 4.8: The predictive value of cytoplasmic MMP-9 in identifying patients who will develop cancer recurrence

Receiver-operator-characteristic curve demonstrating the predictive value of cytoplasmic MMP-9 *in identifying patients who will develop cancer recurrence.*

4.3.3.2 Univariable overall survival - cytoplasmic MMP-9 expression

Cytoplasmic MMP-9 measurements were not statistically higher in patients who died during the follow up period (p=0.206, Mann-Whitney U, figure 4.9). The median for patients who died was 40.00 (IQR 0.00 – 83.33) compared with 35.00 (IQR 0.00 – 78.33) in the alive group.

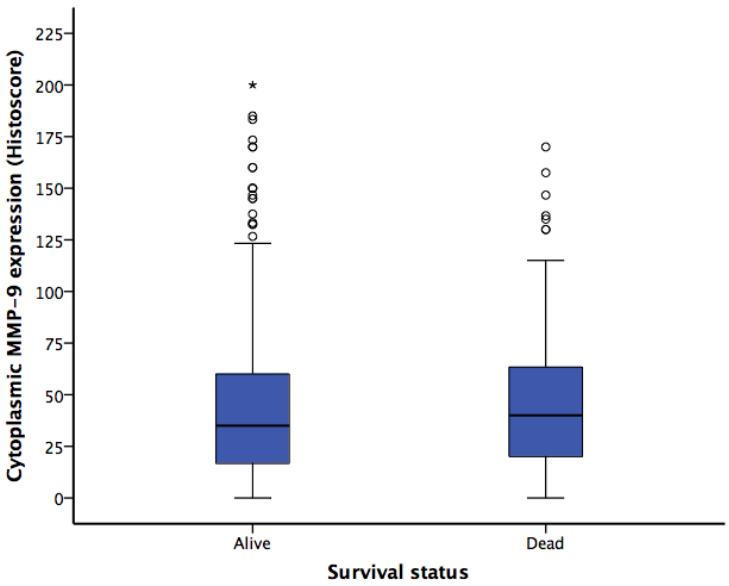
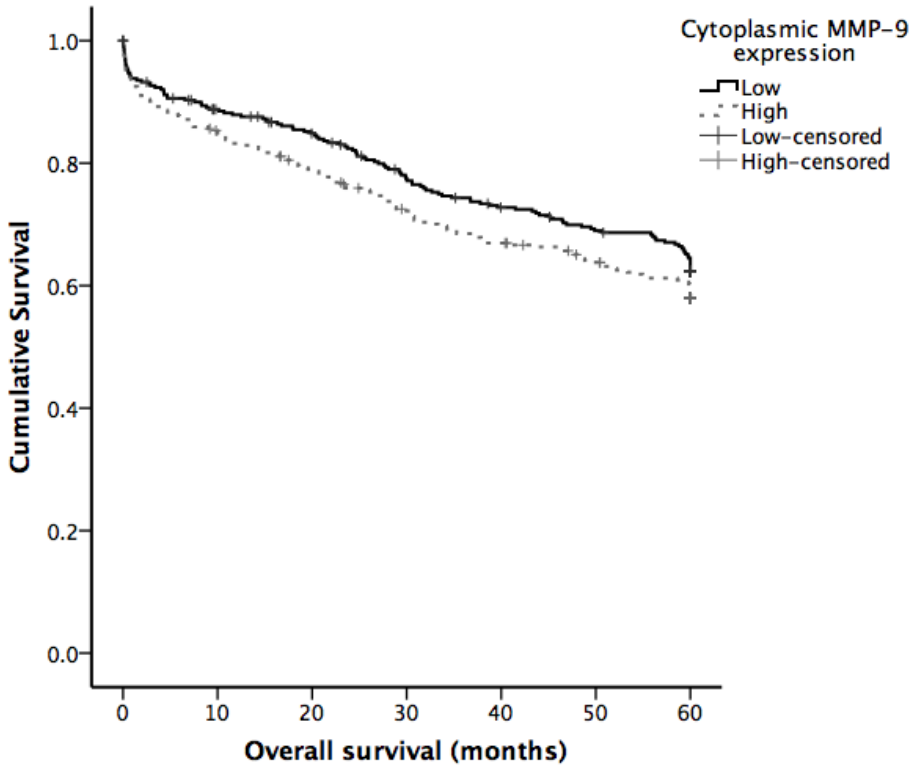


Figure 4.9: The distribution of cytoplasmic MMP-9 measurements in patients stratified by survival status (p=0.206)

The 5 year overall survival rate for patients with a raised cytoplasmic MMP-9 expression was 55.7% compared to 59.0% in patients with a low cytoplasmic MMP-9 expression (p=0.750, Pearson's chi square). On logrank analysis, raised cytoplasmic MMP-9 expression was not significantly associated with poor overall survival (p=0.177, figure 4.10). The mean survival for patients with a raised cytoplasmic MMP-9 expression was 44.3 months (95% CI 41.9 – 46.7) compared with 47.4 months (95% CI 45.2 – 49.6) in the low cytoplasmic MMP-9 expression group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Low	343	295	277	249	231	218	203
High	334	282	258	233	216	201	187

Figure 4.10: The relationship between cytoplasmic MMP-9 expression and overall survival in patients with colorectal cancer (p=0.177)

Kaplan-Meier curve demonstrating the proportion of patients surviving stratified by cytoplasmic MMP-9 *expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.*

On Cox univariable regression analysis raised cytoplasmic MMP-9 was not significantly associated with poor overall survival when dichotomised as a categorical variable (HR 1.18 (95% CI 0.93 – 1.51), p=0.178) or when analysed as a continuous variable (HR 1.00 95% CI 1.00 – 1.00, p=0.634). In addition to the non-significant associations with survival, cytoplasmic MMP-9 did not predict death during follow-up as a categorical variable (AUC

was 0.53 (95% CI 0.48 – 0.57, p=0.206), ROC analysis) or as a continuous variable (AUC was 0.53 (95% CI 0.48 – 0.57, p=0.233), ROC analysis), (figure 4.11).

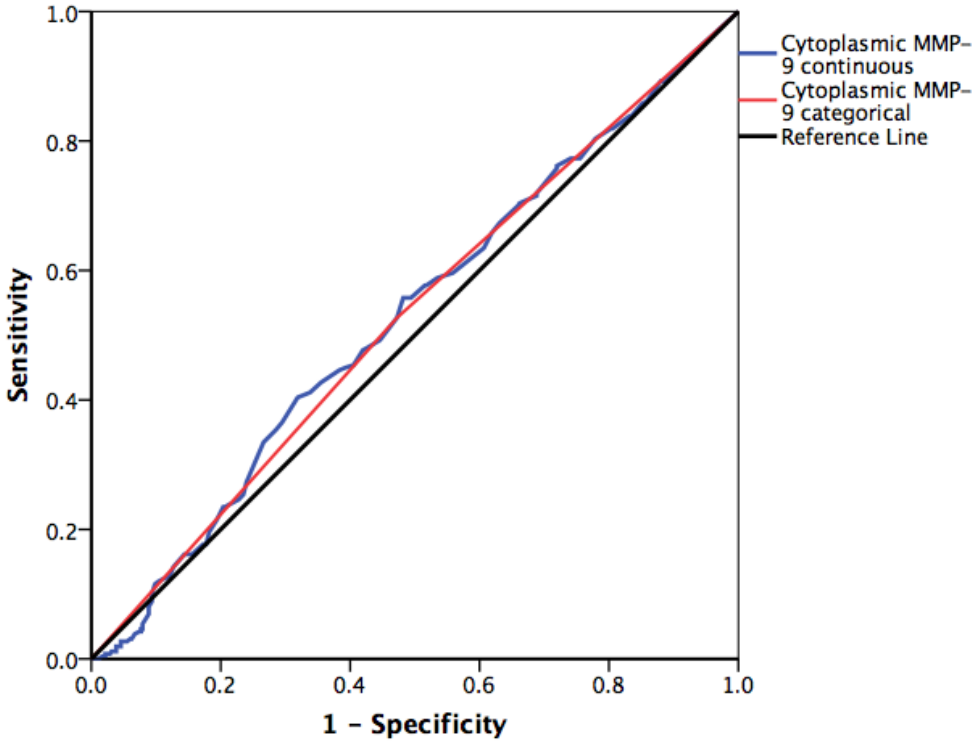


Figure 4.11: Predictive value of cytoplasmic MMP-9 in identifying patients who will die during follow-up

Receiver-operator-characteristic curve demonstrating the predictive value of cytoplasmic MMP-9 *in identifying patients who will die during follow-up.*

4.3.4 Association of cytoplasmic MMP-9 expression, clinicopathological factors stratified by TNM stage

4.3.4.1 The relationship between cytoplasmic MMP-9 expression and TNM stage

There was no significant difference in the measurements of cytoplasmic MMP-9 between patients with TNM stage II and III colorectal cancer (p=0.436, Mann-Whitney U, figure 4.12). The cytoplasmic MMP-9 measurements in patients with stage II colorectal cancer ranged from 0.00 – 185.00 with a median measurement of 40.00 (IQR 0.00 – 85.00) compared with a range of 0.00 – 200.00 and a median measurement of 37.50 (IQR 0.00 – 82.50) in patients with stage III colorectal cancer. This observation was also supported by chi-squared analysis when cytoplasmic MMP-9 was analysed as a categorical variable (p=0.462, Bonferroni adjustment p<0.0125). One hundred and nineteen (41.3%) high

cytoplasmic MMP-9 expressers were TNM stage III compared to 126 (44.4%) of low expressers.

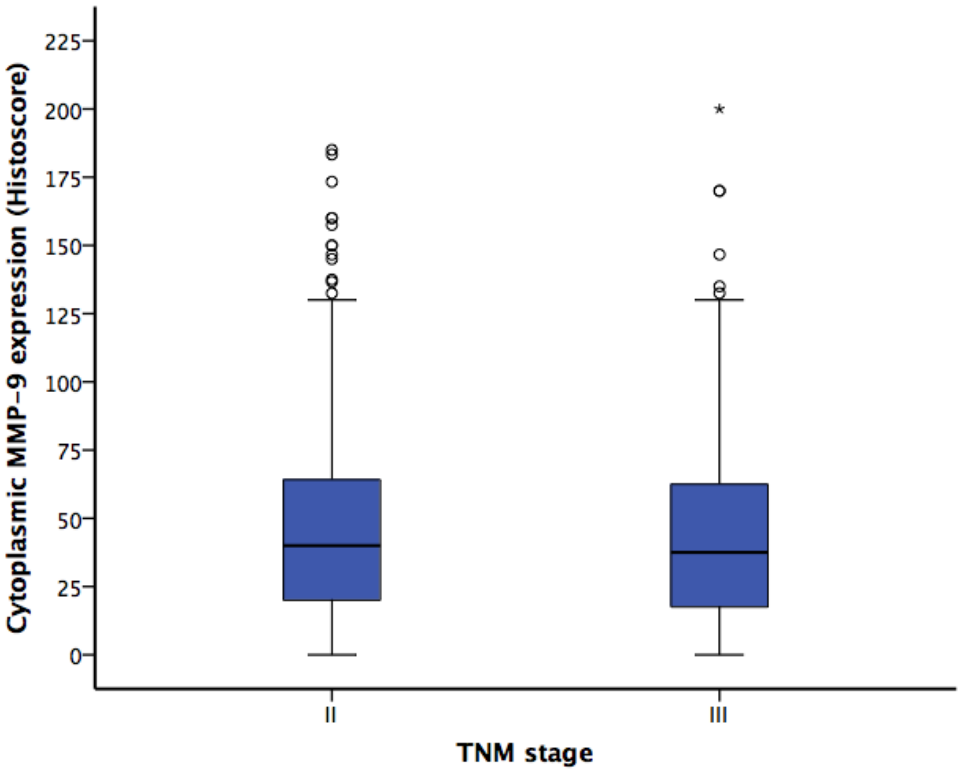


Figure 4.12: Distribution of measurements of cytoplasmic MMP-9 expression in patients with TNM stage II and III colorectal cancer (p=0.436)

4.3.5 Association of cytoplastmic MMP-9 expression, clinicopathological factors and survival – TNM stage II

During follow-up there were 54 (16.5%) recurrences and 114 (34.9%) deaths. Five year recurrence-free and overall survival rates for the clinicopathological factors studied can be found in appendix 3.3.

4.3.5.1 Univariable recurrence-free survival - cytoplasmic MMP-9 expression

Cytoplasmic MMP-9 measurements were not significantly different in patients who went on to develop disease recurrence (p=0.414, Mann-Whitney U, figure 4.13). The median measurement for patients with recurrence was 36.25 (IQR 0.00 – 73.75) compared with 40.00 (IQR 0.00 – 86.25) in the non-recurrence group.

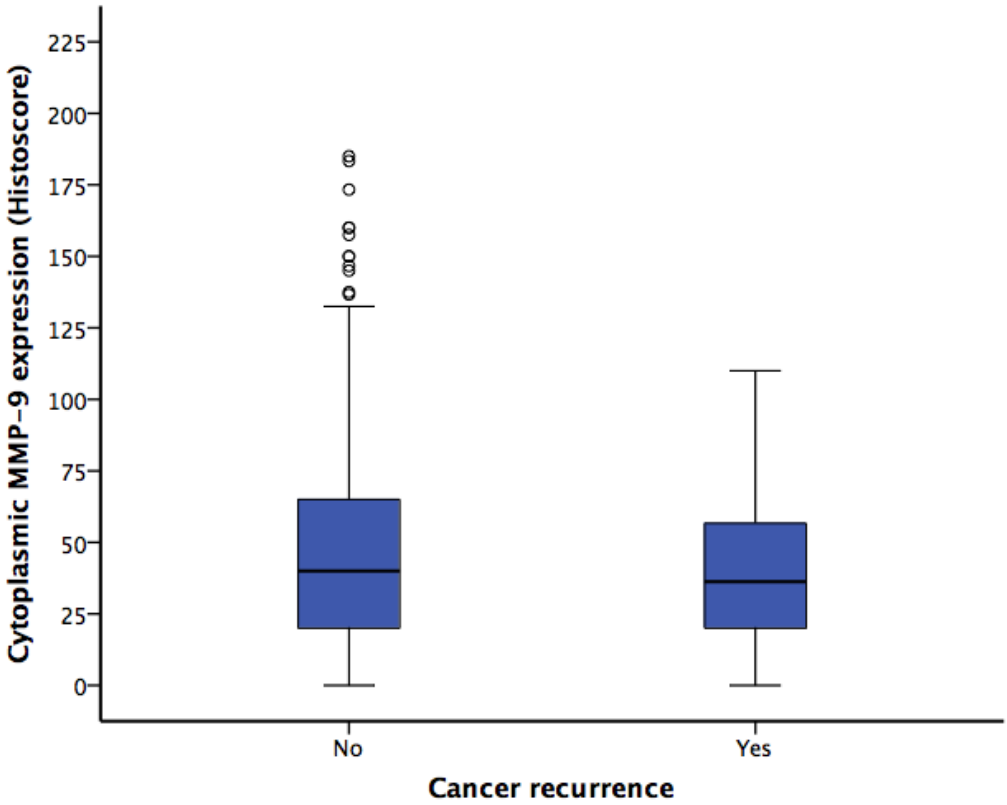
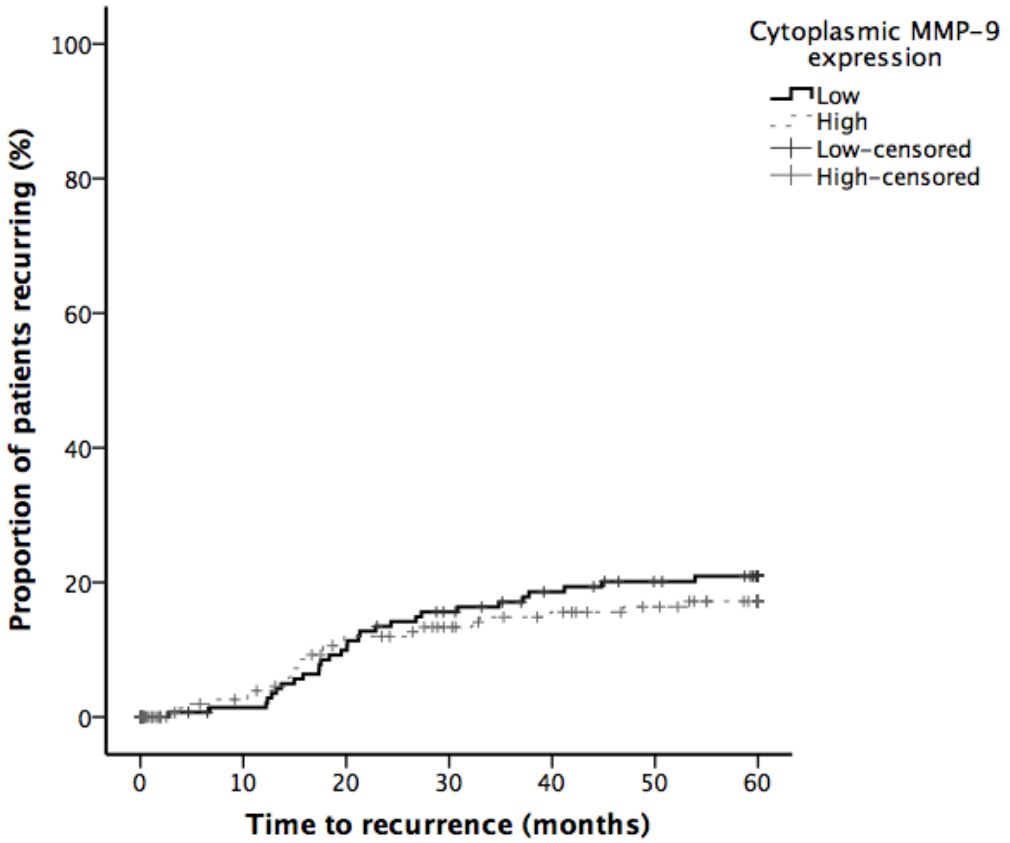


Figure 4.13: The distribution of cytoplasmic MMP-9 measurements in patients with and without cancer recurrence in patients with stage II colorectal cancer (p=0.414)

The 5 year recurrence-free survival rate for patients with a raised cytoplasmic MMP-9 was 56.8% compared to 60.0% in patients with a low cytoplasmic MMP-9 (p=0.826, Pearson's chi square). On logrank analysis raised cytoplasmic MMP-9 was not significantly associated with poor recurrence free survival (p=0.483, figure, 4.14). The mean survival for patients with a raised cytoplasmic MMP-9 was 53.2 months (95% CI 50.6 – 55.7) compared with 52.3 months (95% CI 49.6 – 54.9) in the low cytoplasmic MMP-9 group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	158	139	127	116	107	101	95
High	169	148	129	120	112	105	95

Figure 4.14: The relationship between cytoplasmic MMP-9 expression and recurrence-free survival in patients with stage II colorectal cancer (p=0.483)

Kaplan-Meier curve demonstrating the proportion of patients recurring stratified by cytoplasmic MMP-9 *expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.*

Cox univariable regression analysis demonstrated that a raised cytoplasmic MMP-9 expression was not significantly associated with recurrence-free survival when dichotomised as a categorical variable (HR 0.83 (95% CI 0.48 – 1.41), p=0.483) or as a continuous variable (HR 1.00 (95% CI 0.99 – 1.00), p=0.291). Furthermore, raised cytoplasmic MMP-9 did not predict cancer recurrence as a continuous variable (AUC of 0.47 (95% CI 0.39 – 0.55, p=0.414)) or as a categorical variable (AUC of 0.47 (95% CI 0.38 – 0.55, p=0.454) (figure 4.15).

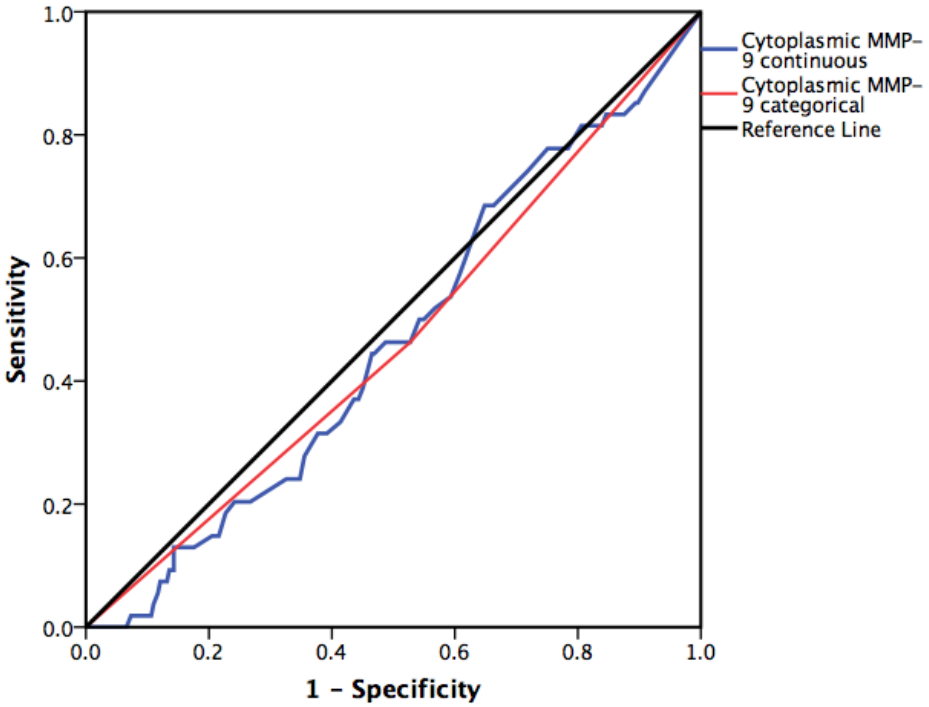


Figure 4.15: The predictive value of cytoplasmic MMP-9 in identifying patients with stage II colorectal cancer who will develop recurrence during follow-up

Receiver-operator-characteristic curve demonstrating the predictive value of cytoplasmic MMP-9 *in identifying patients who will survive 5 years without recurrence.*

4.3.5.2 Univariable overall survival - cytoplasmic MMP-9 expression

Cytoplasmic MMP-9 measurements were not significantly higher in patients who died during the follow up period (p=0.282, Mann-Whitney U, figure 4.16). The median for patients who died was 45.00 (IQR 0.00 – 95.00) compared with 37.50 (IQR 0.00 – 78.75) in the alive group.

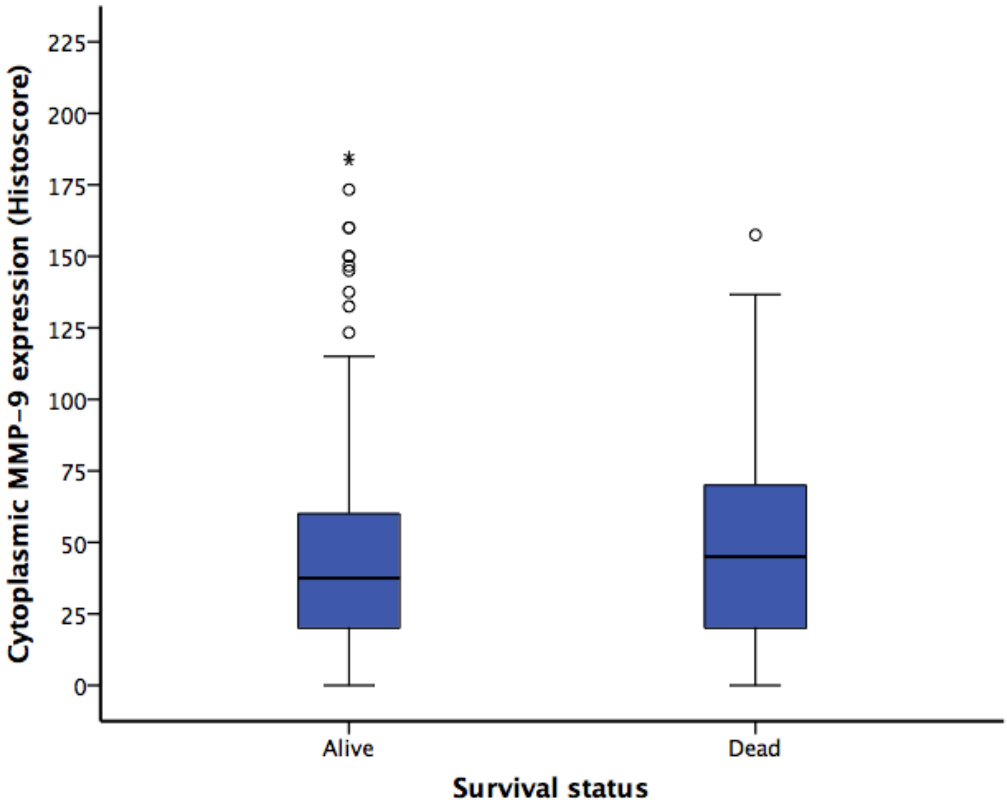
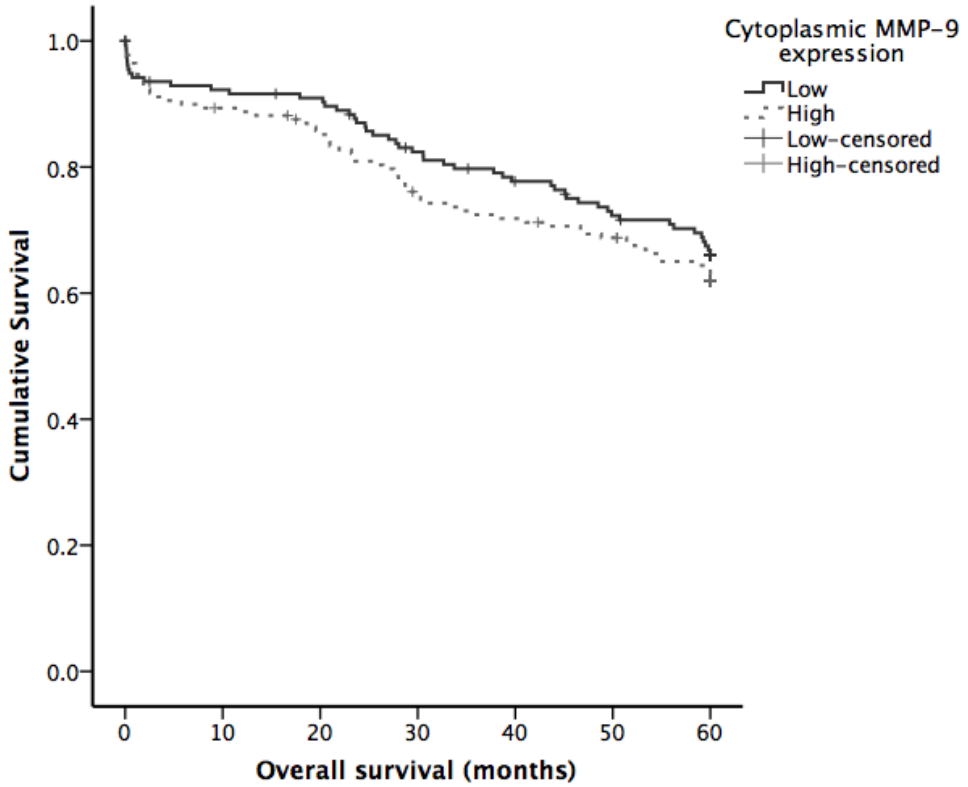


Figure 4.16: The distribution of cytoplasmic MMP-9 measurements in patients stratified by survival status (p=0.282)

The 5 year overall survival rate for patients with a raised cytoplasmic MMP-9 was 60.4% compared to 61.4% in patients with a normal serum CRP (p=0.992, Pearson's chi square). On logrank analysis raised cytoplasmic MMP-9 expression was not significantly associated with poor overall survival (p=0.366, figure 4.17). The mean survival for patients with a raised cytoplasmic MMP-9 expression was 47.0 months (95% CI 43.9 – 50.1) compared with 49.8 months (95% CI 46.8 – 52.7) in the low cytoplasmic MMP-9 group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	158	142	139	124	115	106	97
High	169	150	141	124	118	112	102

Figure 4.17: The relationship between cytoplasmic MMP-9 expression and overall survival in patients with stage II colorectal cancer

Kaplan-Meier curve demonstrating the proportion of patients surviving stratified by cytoplasmic MMP-9 *expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.*

On Cox univariable regression analysis raised cytoplasmic MMP-9 was not associated with overall survival when dichotomised as a categorical variable (HR 1.19 (95% CI 0.82 – 1.71), p=0.367) or as a continuous variable (HR 1.00 95% CI 1.00 – 1.01, p=0.619). Furthermore, there was no predictive association between cytoplasmic MMP-9 expression and survival when ROC analysis was performed using death as the endpoint. The AUC was 0.54 (95% CI 0.47 – 0.60, p=0.283) for cytoplasmic MMP-9 as a continuous variable and an AUC of 0.53 (95% CI 0.46 – 0.59, p=0.413) for cytoplasmic MMP-9 as a categorical variable (figure 4.18).

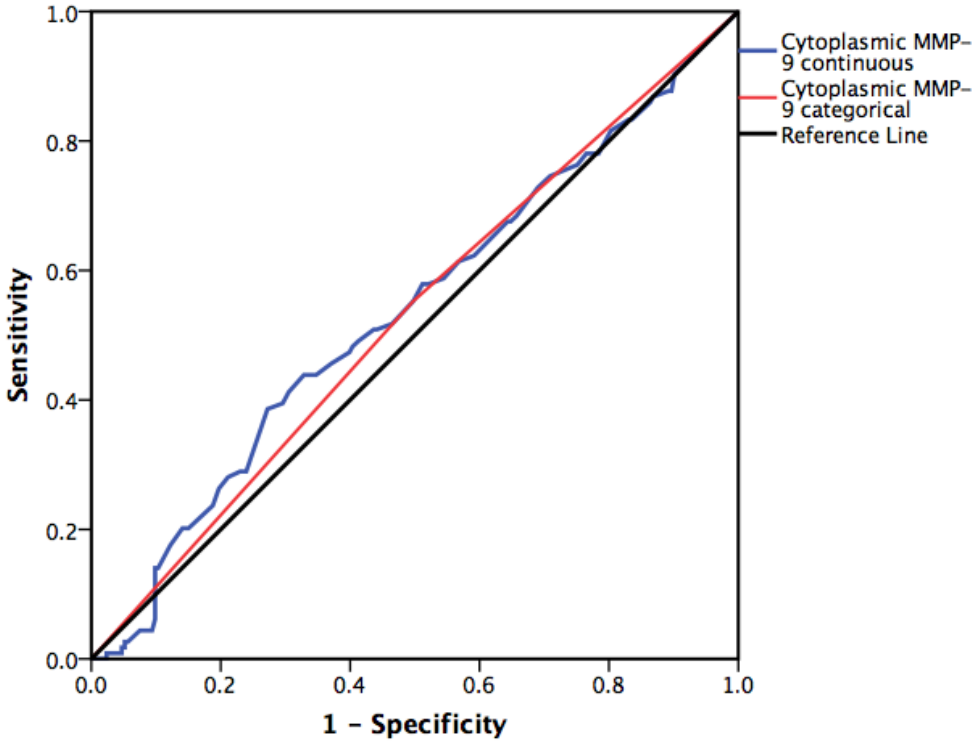


Figure 4.18: The predictive value of cytoplasmic MMP-9 in identifying patients with stage II colorectal cancer who will die during follow-up

Receiver-operator-characteristic curve demonstrating the predictive value of cytoplasmic MMP-9 *in identifying patients who will die during follow-up.*

4.3.6 Association of cytoplasmic MMP-9 expression, clinicopathological factors and survival – TNM stage III

During follow-up there were 89 (36.3%) recurrences and 121 (49.4%) deaths. Five year recurrence free and overall survival rates for the clinicopathological factors studied can be found in appendix 3.4.

4.3.6.1 Univariable recurrence-free survival - cytoplasmic MMP-9 expression

Cytoplasmic MMP-9 measurements were not significantly different in patients who went on to develop disease recurrence (p=0.897, Mann-Whitney U, figure 4.19). The median measurement for patients with recurrence was 37.50 (IQR 0.00 – 130.00) compared with 37.09 (IQR 0.00 – 82.09) in the non-recurrence group.

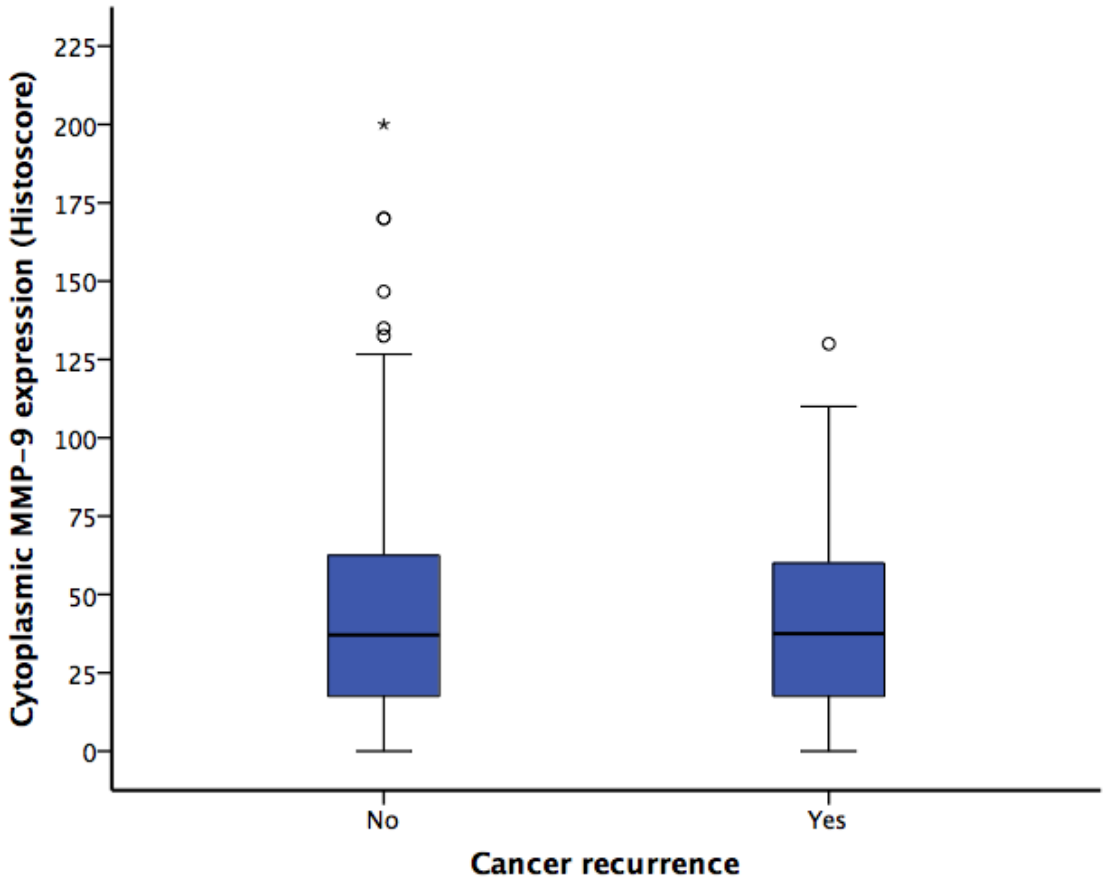
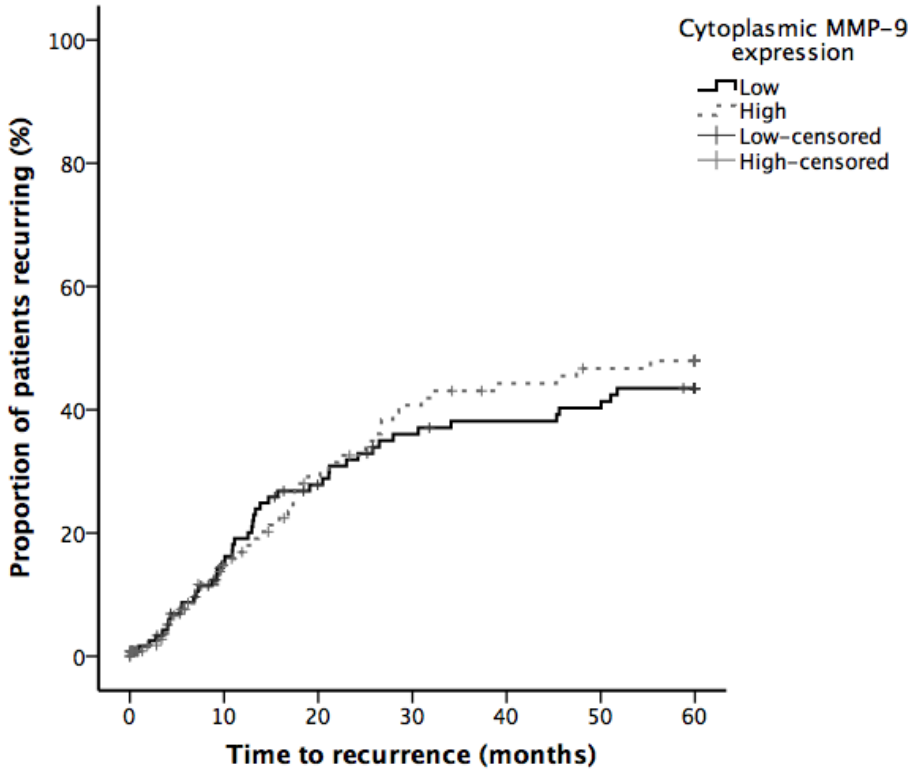


Figure 4.19: The distribution of cytoplasmic MMP-9 measurements in patients with and without cancer recurrence in patients with stage III colorectal cancer (p=0.897)

The 5 year recurrence free survival rate for patients with a raised cytoplasmic MMP-9 was 35.3% compared to 41.3% in patients with a low cytoplasmic MMP-9 (p=0.473, Pearson's chi square). On logrank analysis raised cytoplasmic MMP-9 was not significantly associated with poor recurrence-free survival (p=0.652, figure 4.20). The mean survival for patients with a raised cytoplasmic MMP-9 was 40.1 months (95% CI 35.6 – 44.7) compared with 41.6 months (95% CI 37.3 – 46.0) in the low cytoplasmic MMP-9 group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	126	88	71	61	58	56	52
High	119	80	62	51	46	43	42

Figure 4.20: The relationship between cytoplasmic MMP-9 expression and recurrence-free survival in patients with stage III colorectal cancer (p=0.652)

Kaplan-Meier curve demonstrating the proportion of patients recurring stratified by cytoplasmic MMP-9 expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

Cox univariable regression analysis demonstrated that a raised cytoplasmic MMP-9 expression was not significantly associated with poor recurrence-free survival when dichotomised as a categorical variable (HR 1.10 (95% CI 0.73 – 1.67), p=0.652) or as a continuous variable (HR 1.00 (95% CI 0.99 – 1.01), p=0.875). Furthermore, raised cytoplasmic MMP-9 did not predict cancer recurrence as a continuous variable (AUC of 0.50 (95% CI 0.42 – 0.57, p=0.897)) or as a categorical variable (AUC of 0.51 (95% CI 0.43 – 0.58, p=0.859) (figure 4.21).

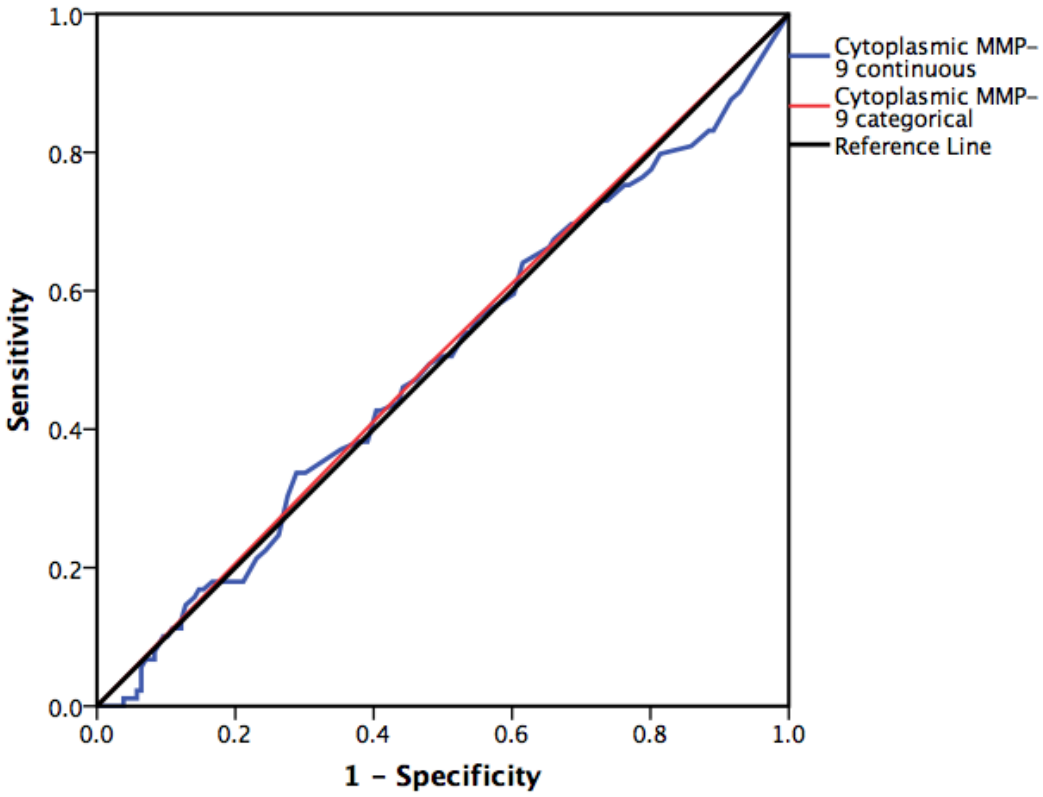


Figure 4.21: The predictive value of cytoplasmic MMP-9 in identifying patients with stage III colorectal cancer who will develop recurrence during follow-up

(A) Receiver-operator-characteristic curve demonstrating the predictive value of cytoplasmic MMP-9 in identifying patients who will survive 5 years without recurrence.

4.3.6.2 Univariable overall survival - cytoplasmic MMP-9 expression

Cytoplasmic MMP-9 measurements were not significantly higher in patients who died during follow-up (p=0.324, Mann-Whitney U, figure 4.22). The median for patients who died was 40.00 (IQR 0.00 – 82.50) compared with 33.54 (IQR 0.00 – 78.12) in the alive group.

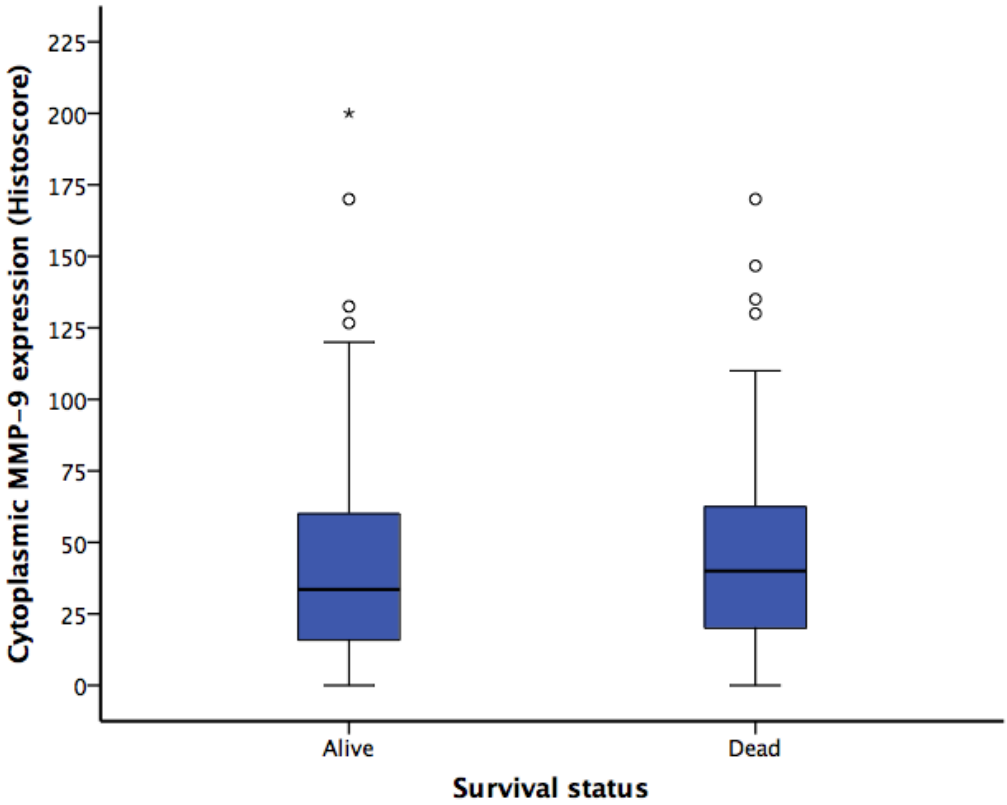
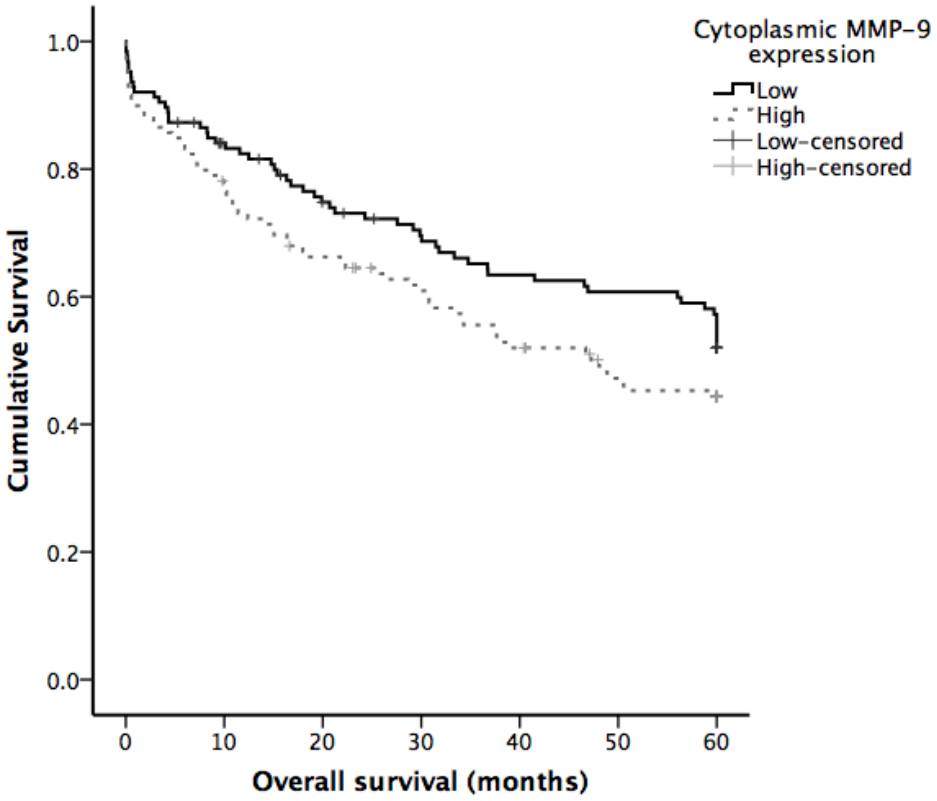


Figure 4.22: The distribution of cytoplasmic MMP-9 measurements in patients stratified by survival status (p=0.324)

The 5 year overall survival rate for patients with a raised cytoplasmic MMP-9 was 38.7% compared to 51.6% in patients with a low cytoplasmic MMP-9 (p=0.127, Pearson's chi square). On logrank analysis raised cytoplasmic MMP-9 was not significantly associated with poor overall survival (p=0.140, figure 4.23). The mean survival for patients with a raised cytoplasmic MMP-9 expression was 37.2 months (95% CI 32.8 – 41.6) compared with 42.9 months (95% CI 38.8 – 47.0) in the low cytoplasmic MMP-9 group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	126	101	87	79	72	69	65
High	119	91	77	69	58	49	47

Figure 4.23: The relationship between cytoplasmic MMP-9 expression and overall survival in patients with stage III colorectal cancer (p=0.140)

Kaplan-Meier curve demonstrating the proportion of patients surviving stratified by cytoplasmic MMP-9 *expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.*

On Cox univariable regression analysis raised cytoplasmic MMP-9 was not associated with overall survival when dichotomised as a categorical variable (HR 1.31 (95% CI 0.91 – 1.87), p=0.142) or as a continuous variable (HR 1.00 95% CI 1.00 – 1.01, p=0.407). Furthermore, there was no predictive association between cytoplasmic MMP-9 expression and survival when ROC analysis was performed using death as the endpoint. The AUC was 0.54 (95% CI 0.46 – 0.61, p=0.324) for cytoplasmic MMP-9 as a continuous variable and an AUC of 0.54 (95% CI 0.47 – 0.62, p=0.248) for cytoplasmic MMP-9 as a categorical variable (figure 4.24).

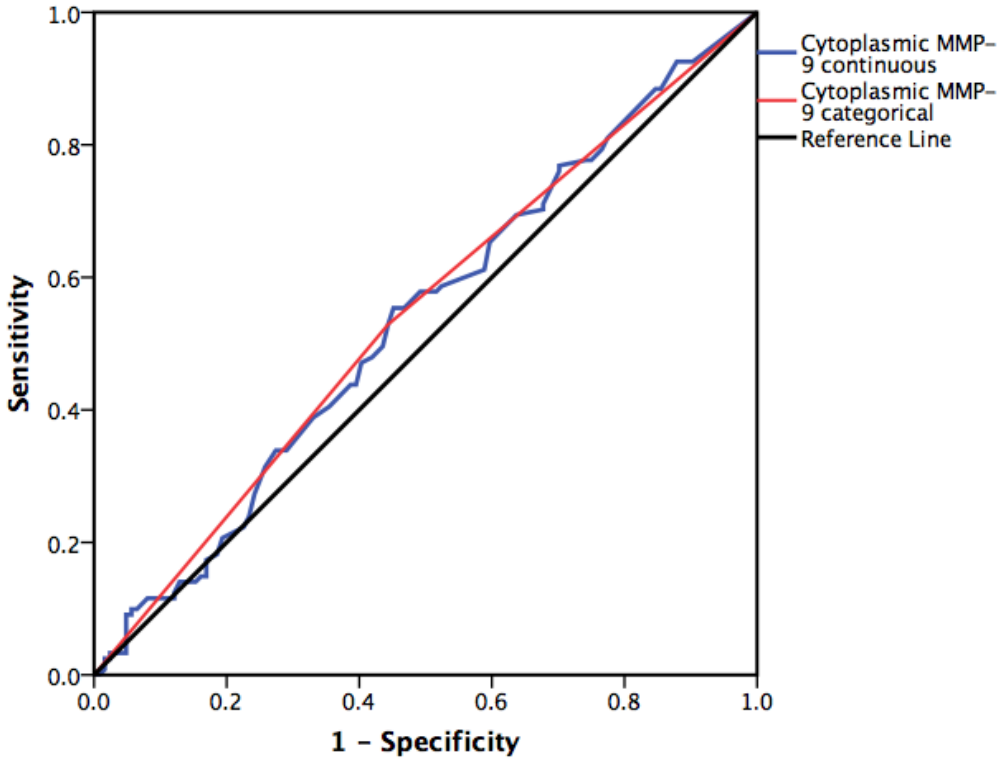


Figure 4.24: The predictive value of cytoplasmic MMP-9 in identifying patients with stage III colorectal cancer who will die during follow-up

Receiver-operator-characteristic curve demonstrating the predictive value of cytoplasmic MMP-9 in identifying patients who will die during follow up.

4.3.7 Association of cytoplasmic MMP-9 expression and clinicopathological factors stratified by MSI status

There was no significant difference in the measurements of cytoplasmic MMP-9 between patients with CI and MSI colorectal cancer (p=0.919, Mann-Whitney U, figure 4.25). The cytoplasmic MMP-9 measurements in patients with CI colorectal cancer ranged from 0.00 – 200.00 with a median of 37.50 (IQR 0.00 – 81.88) compared with a range of 0.00 – 200.00 and a median of 33.33 (IQR 0.00 – 89.17) in patients with MSI colorectal cancer. This non-relationship was also observed by chi-squared analysis when cytoplasmic MMP-9 was analysed as a categorical variable (p=0.822, table 4.1).

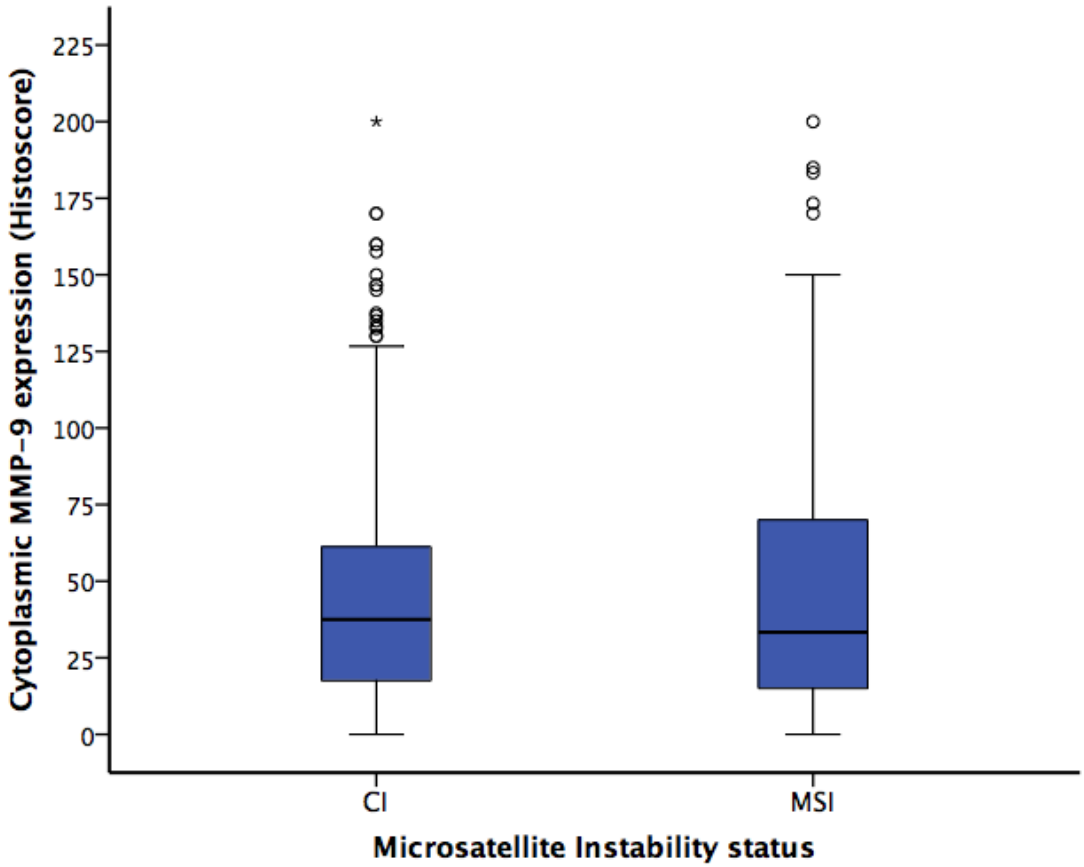


Figure 4.25: Distribution of measurements of cytoplasmic MMP-9 expression in patients with CI and MSI colorectal cancer (p=0.919)

4.3.8 Association of cytoplasmic MMP-9 expression, clinicopathological factors and survival – CI colorectal cancer

During follow-up there were 140 (23.6%) recurrences and 227 (38.3%) deaths. Five year recurrence free and overall survival rates for the clinicopathological factors studied can be found in appendix 3.5.

4.3.8.1 Univariable recurrence-free survival - cytoplasmic MMP-9 expression

Cytoplasmic MMP-9 measurements were not significantly different in patients who went on to develop disease recurrence (p=0.480, Mann-Whitney U, figure 4.26). The median measurement for patients with recurrence was 37.50 (IQR 0.00 – 80.00) compared with 40.00 (IQR 0.00 – 85.00) in the non-recurrence group.

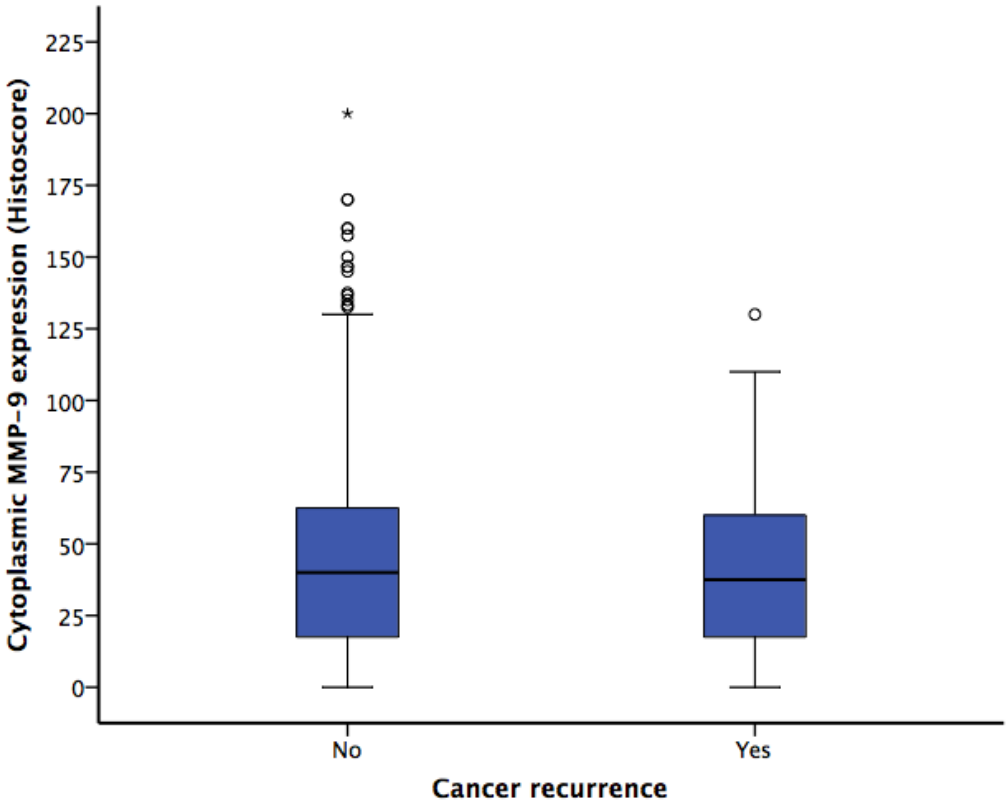
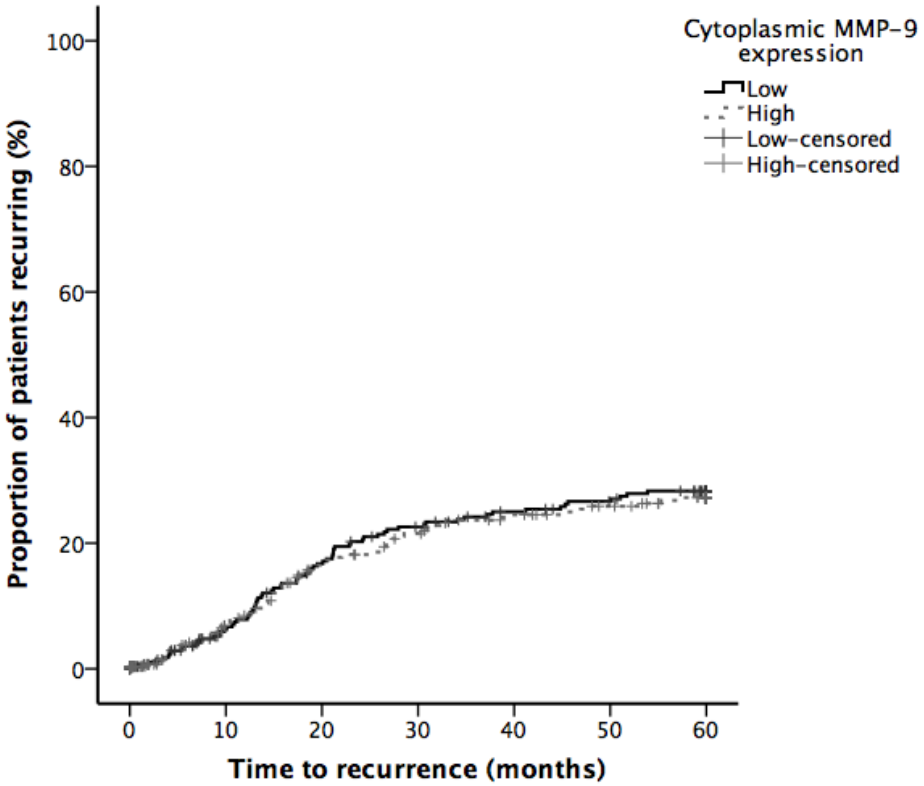


Figure 4.26: The distribution of cytoplasmic MMP-9 measurements in patients with and without cancer recurrence in patients with CI colorectal cancer (p=0.480)

The 5 year recurrence-free survival rate for patients with a raised cytoplasmic MMP-9 was 51.7% compared to 55.4% in patients with a low cytoplasmic MMP-9 (p=0.727, Pearson's chi square). On logrank analysis raised cytoplasmic MMP-9 was not significantly associated with poor recurrence free survival (p=0.798, figure 4.27). The mean survival for patients with a raised cytoplasmic MMP-9 was 49.1 months (95% CI 46.7 – 51.5) compared with 48.6 months (95% CI 46.3 – 51.0) in the low cytoplasmic MMP-9 group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	298	243	214	196	184	177	165
High	294	236	201	186	173	164	152

Figure 4.27: The relationship between cytoplasmic MMP-9 expression and recurrence-free survival in patients with CI colorectal cancer (p=0.798)

Kaplan-Meier curve demonstrating the proportion of patients recurring stratified by cytoplasmic MMP-9 expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

Cox univariable regression analysis demonstrated that a raised cytoplasmic MMP-9 expression was not significantly associated with poor recurrence-free survival when dichotomised as a categorical variable (HR 0.96(95% CI 0.69 – 1.33), p=0.798) or as a continuous variable (HR 1.00 (95% CI 0.99 – 1.00), p=0.477). Furthermore, raised cytoplasmic MMP-9 did not predict cancer recurrence as a continuous variable (AUC of 0.48 (95% CI 0.43 – 0.54, p=0.480)) or as a categorical variable (AUC of 0.49 (95% CI 0.43 – 0.54, p=0.672) (figure 4.28).

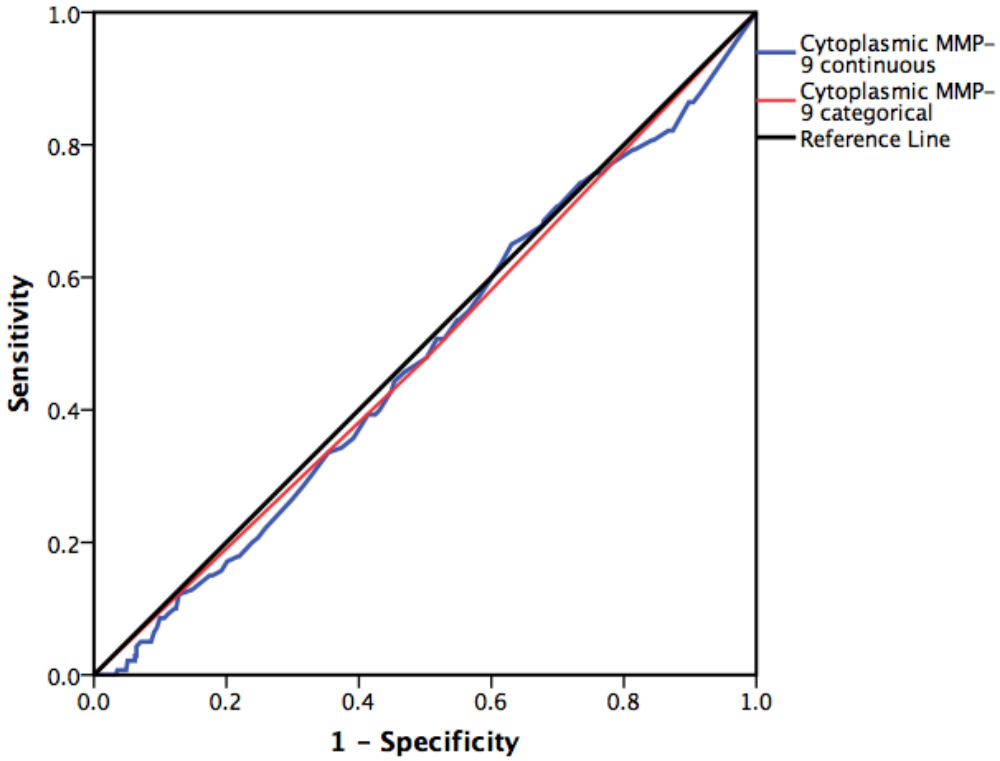


Figure 4.28: The predictive value of cytoplasmic MMP-9 in identifying patients with CI colorectal cancer who will develop recurrence during follow-up

Receiver-operator-characteristic curve demonstrating the predictive value of cytoplasmic MMP-9 in identifying patients who will survive 5 years without recurrence.

4.3.8.2 Univariable overall survival - cytoplasmic MMP-9 expression

Cytoplasmic MMP-9 measurements were not significantly higher in patients who died during follow-up (p=0.086, Mann-Whitney U, figure 4.29). The median measurement for patients who died was 42.50 (IQR 0.00 – 87.50) compared with 35.00 (IQR 0.00 – 78.54) in the alive group.

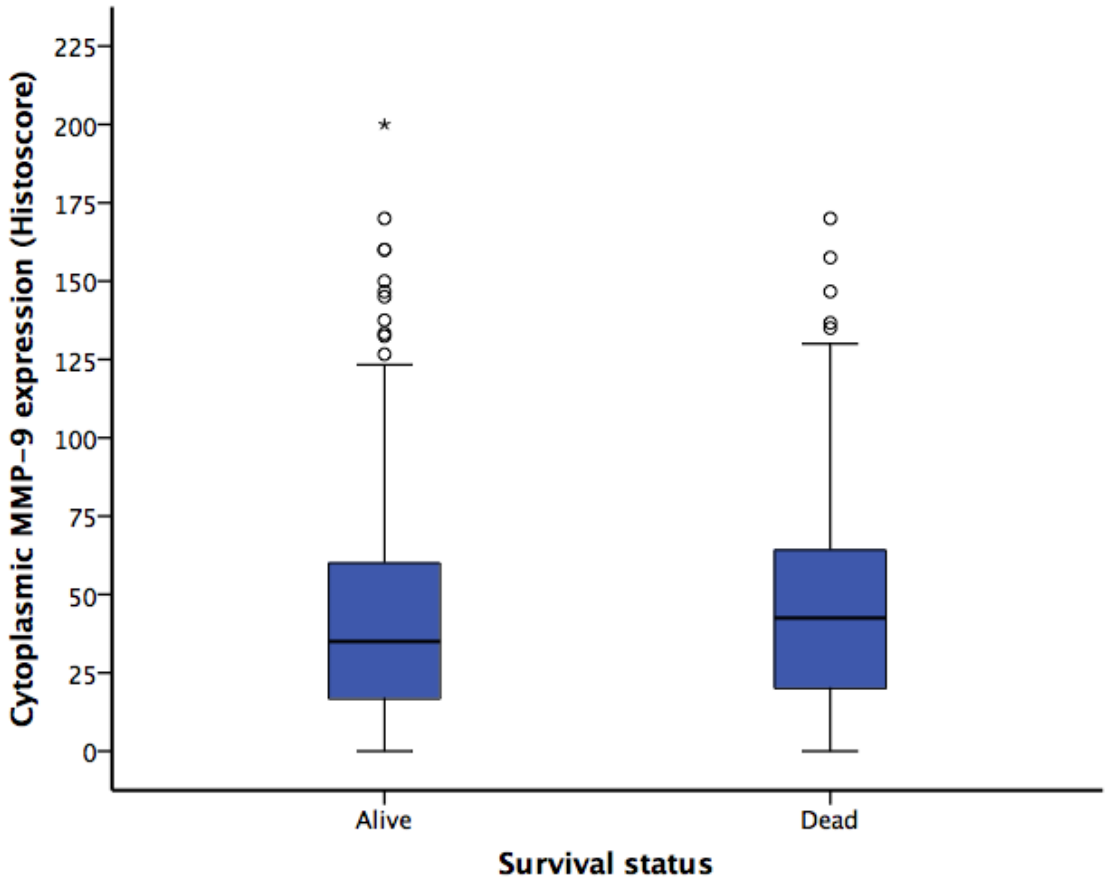
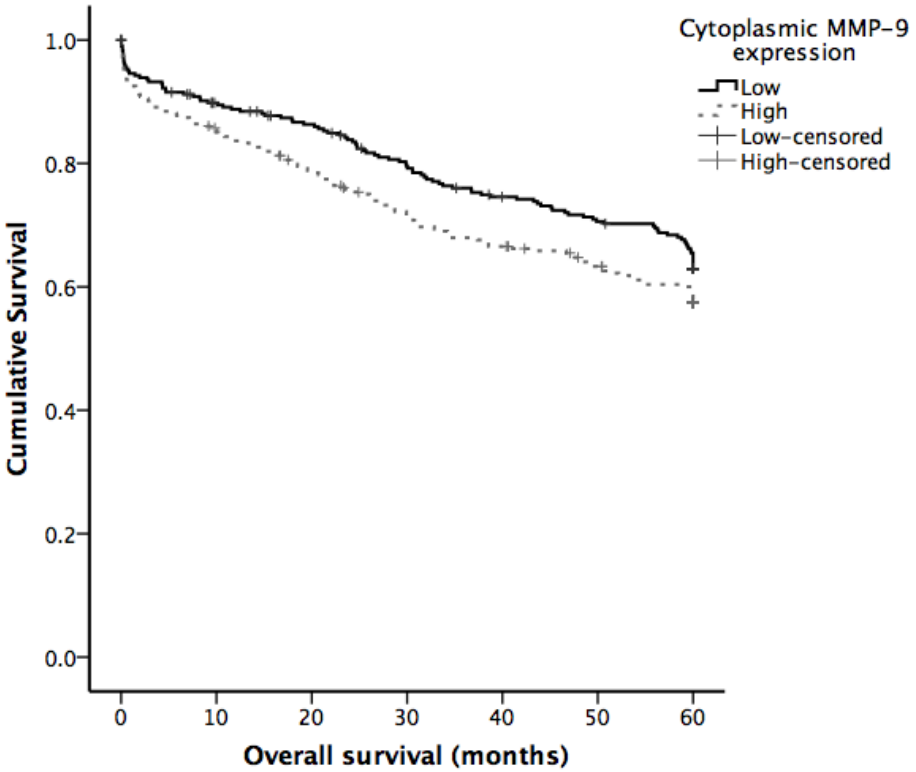


Figure 4.29: The distribution of cytoplasmic MMP-9 measurements in patients stratified by survival status (p=0.086)

The 5 year overall survival rate for patients with a raised cytoplasmic MMP-9 was 55.1% compared to 60.4% in patients with a normal serum MMP-9 (p=0.618, Pearson's chi square). On logrank analysis raised cytoplasmic MMP-9 was not significantly associated with poor overall survival (p=0.098, figure 4.30). The mean survival for patients with a raised cytoplasmic MMP-9 expression was 44.1 months (95% CI 41.6 – 46.7) compared with 48.2 months (95% CI 45.9 – 50.5) in the low cytoplasmic MMP-9 group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	298	259	245	222	206	195	180
High	294	249	226	204	189	175	163

Figure 4.30: The relationship between cytoplasmic MMP-9 expression and overall survival in patients with CI colorectal cancer (p=0.098)

Kaplan-Meier curve demonstrating the proportion of patients surviving stratified by cytoplasmic MMP-9 expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis raised cytoplasmic MMP-9 was not associated with overall survival when dichotomised as a categorical variable (HR 1.25 (95% CI 0.96 – 1.62), p=0.099) or as a continuous variable (HR 1.00 95% CI 1.00 – 1.01, p=0.100). Furthermore, there was no predictive association between cytoplasmic MMP-9 expression and survival when ROC analysis was performed using death as the endpoint. The AUC was 0.54 (95% CI 0.49 – 0.59, p=0.086) for cytoplasmic MMP-9 as a continuous variable and an AUC of 0.53 (95% CI 0.49 – 0.58, p=0.175) for cytoplasmic MMP-9 as a categorical variable (figure 4.31).

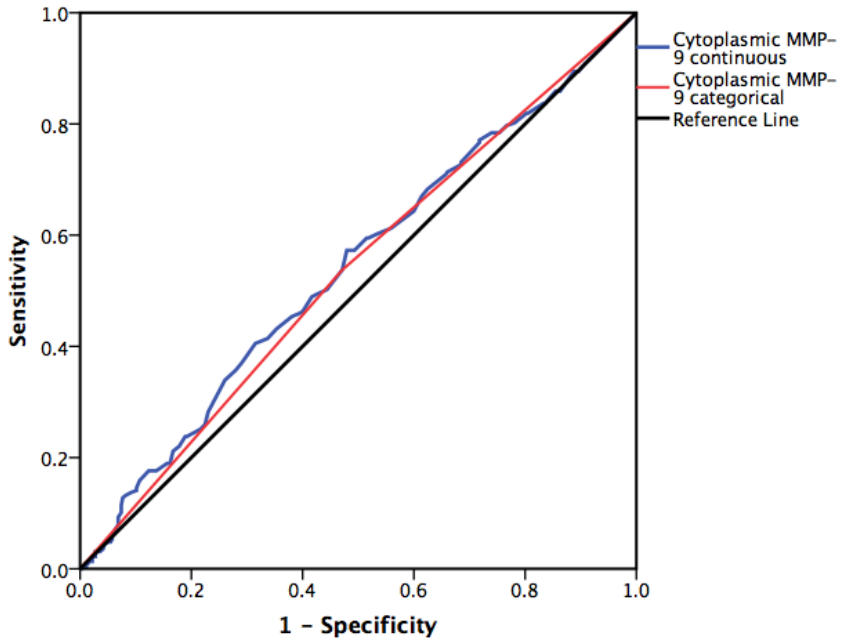


Figure 4.31: The predictive value of cytoplasmic MMP-9 in identifying patients with CI colorectal cancer who will die during follow-up

Receiver-operator-characteristic curve demonstrating the predictive value of cytoplasmic MMP-9 *in identifying patients who will die during follow-up.*

4.3.8.3 Multivariable recurrence-free survival

Cytoplasmic MMP-9 expression was not associated with recurrence-free survival and therefore, no new multivariable model including this biomarker has been constructed.

4.3.8.4 Multivariable overall survival

Proportionality assumptions testing

All the studied variables underwent Cox proportional hazards assumption testing as described previously. Age, presentation and serum albumin demonstrated time dependency when interaction terms between the covariates and log(time) were placed in the model.

Cox proportional hazards regression analysis

All covariates with a significance level of $p<0.1$ were included in the multivariable model. Terms were removed if their respective p-value was >0.05. On univariable analysis, advancing age ($p<0.001$), emergency presentation ($p<0.001$), advancing T-stage ($p<0.001$), advancing N-stage ($p=0.001$), higher TNM stage ($p<0.001$), poor differentiation ($p<0.001$), raised serum CRP ($p<0.001$), hypoalbuminaemia ($p<0.001$), good Klintrup score ($p<0.001$)

and raised cytoplasmic MMP-9 expression (p=0.099) had a p-value <0.1 and were therefore included in the multivariable Cox proportional hazards regression model.

On multivariable analysis, advancing age (HR 1.05 (95% CI 1.03 – 1.08), p<0.001), emergency presentation (HR 2.09 (95% CI 1.31 – 3.35), p=0.002), T-stage (when all subcategories were compared with T1 (T2 HR 1.18 (95% CI 0.34 – 4.13), (T3 HR 1.34 (95% CI 0.42 – 4.29) and (T4 HR 2.38 (95% CI 0.73 – 7.75)), p=0.004), hypoalbuminaemia (HR 1.49 (95% CI 1.06 – 2.10), p=0.022) and good Klintrup score (HR 0.50 (95% CI 0.33 – 0.75), p=0.001) were independently associated with poor overall survival (table 4.2).

Influence of time dependency on outcome

Only emergency presentation (p=0.018) retained significant time dependency when placed in the multivariable analysis with age (p=0.051) and serum albumin (p=0.174) becoming non-significant.

Table 4.2: The relationships between cytoplasmic MMP-9 expression, clinicopathological factors and overall survival in patients with CI colorectal cancer: univariable and multivariable analysis

	Univariable analysis			Multivariable analysis		
	Coefficient	p-value	Hazard ratio (95% CI)	Coefficient	p-value	Hazard ratio (95% CI)
Age	0.052	<0.001	1.05 (1.03 – 1.08)	0.052	<0.001	1.05 (1.03 – 1.08)
Age x log(time)	-0.009	0.012	0.99 (0.98 - 1.00)	-0.008	0.051	0.99 (0.98 – 1.00)
Sex						
Female			1			
Male	-0.059	0.657	0.94 (0.73 – 1.22)			
Presentation						
Elective			1			1
Emergency	1.135	<0.001	3.11 (2.05 – 4.73)	0.737	0.002	2.09 (1.31 – 3.35)
Presentation x log(time)	-0.175	0.014	0.84 (0.73 – 0.97)	-0.184	0.018	0.83 (0.71 – 0.97)
Tumour site						
Right	(0.000)	0.490	1			
Left	-0.180		0.84 (0.62 – 1.13)			
Rectum	-0.112		0.89 (0.64 – 1.25)			
T stage						
1	(0.000)	<0.001	1	(0.000)	0.004	1
2	-0.067		0.94 (0.38 – 2.33)	0.165		1.18 (0.34 – 4.13)
3	0.342		1.41 (0.67 – 3.20)	0.291		1.34 (0.42 – 4.29)
4	1.003		2.73 (1.23 – 6.23)	0.866		2.38 (0.73 – 7.75)
N stage						
0	(0.000)	<0.001	1		0.096	
1	0.413		1.51 (1.12 – 2.04)			
2	0.905		2.47 (1.72 – 3.55)			
TNM stage						
I	(0.000)	<0.001	1		0.225	
II	0.457		1.58 (0.99 – 2.51)			
III	0.915		2.47 (1.72 – 3.95)			
Differentiation						
Well/Moderate			1		0.114	
Poor	0.715	<0.001	2.05 (1.38 – 3.04)			
Serum CRP						
Normal			1			
High	0.796	<0.001	2.22 (1.64 – 3.00)		0.080	
Serum albumin						
Normal			1			1
Low	1.193	<0.001	3.30 (2.13 – 5.12)	0.399	0.022	1.49 (1.06– 2.10)
albumin x log(time)	-0.188	0.013	0.83 (0.72 – 0.96)		0.174	
Klintrup score						
High			1			1
Low	-0.803	<0.001	0.45 (0.32 – 0.63)	-0.694	0.001	0.50 (0.33 – 0.75)
Cytoplasmic MMP-9						
Low			1			
High	0.220	0.099	1.25 (0.96 – 1.62)		0.259	

4.3.9 Association of cytoplasmic MMP-9 expression, clinicopathological factors and survival – MSI colorectal cancer

During follow-up there were 10 (11.8%) recurrences and 33 (38.3%) deaths. It has been suggested that at least 10 - 25 events are required for each variable in a multivariable model. Based on the number of events noted above, no meaningful results can be generated by undertaking univariable or multivariable survival analysis exclusively on MSI patients. The relatively low number of patients and associated events makes the chance of incurring a type I or type II reporting error highly likely. Therefore, survival analysis has not been performed on the subset of patients with MSI colorectal cancer.

4.4 The relationship between serum and tumour MMP-9 expression, clinicopathological factors and survival

4.4.1 Cohort description

Core characteristics of the training, validation and serum cohort are shown in table 4.3. The serum cohort had a similar proportion of male patients to the training cohort. All patients in the serum cohort presented electively. There was a higher proportion of right sided and stage I tumours in the serum cohort compared to the training and validation cohorts.

Table 4.3: Comparison of proportionality of core clinicopathological factors between the training cohort, validation cohort and matched serum/tissue cohort (cohort 3)

Clinicopathological variables	Training cohort (Cohort 1) n=182	Validation cohort (Cohort 2) n=677	Serum cohort (Cohort 3) n=95
Age			
Median +/- IQR	70 (IQR 54 – 86)	72 (IQR 57 - 87)	69 (IQR 56 – 82)
Sex			
Female	42.3%	49.1%	44.2%
Male	57.7%	50.9%	55.8%
Mode of presentation			
Elective	95.6%	70.4%	100.0%
Emergency	4.4%	29.6%	0.0%
Tumour Site			
Colon	67.6%	78.2%	75.8%
Rectum	32.4%	21.8%	24.2%
Tumour site enhanced			
Right	37.9%	44.6%	48.4%
Left	29.7%	34.4%	28.4%
Rectum	32.4%	21.0%	23.2%
Tumour Stage			
I	6.6%	15.7%	18.8%
II	47.3%	48.6%	47.4%
III	46.2%	35.8%	33.7%
IV	0.0%	0.0%	0.0%

4.4.2 Experimental clinicopathological factors

In addition to the basic patient demographics and tumour characteristics presented in section 4.4.1, tumour differentiation, serum CRP and serum albumin was also evaluated. Data on Klintrup score and MMR protein expression was not available for this cohort. All of the 95 patients in the serum cohort (cohort 3) had data on serum CRP and Albumin available. In this cohort, 95.8% of patients were considered well/moderately differentiated. In addition, 26.3% of patients had a raised serum CRP measurement and 32.6% were hypoalbuminaemic (table 4.4).

Table 4.4: Comparison of proportionality of experimental clinicopathological factors between the training cohort, validation cohort matched serum/tissue cohort (cohort 3)

Clinicopathological variables	Training cohort (Cohort 1) n=182	Validation cohort (Cohort 2) n=677	Serum cohort (Cohort 3) n=95
Differentiation			
Well/Moderate	89.0%	90.3%	95.8%
Poor	11.0%	9.7%	4.2%
Serum CRP			
Normal	54.9%	50.7%	73.7%
High	45.1%	49.3% n=533	26.3%
Serum albumin			
Normal	86.3%	73.4%	67.4%
Low	13.7%	26.6% n=563	32.6%
Klintrup score			
High	31.5%	30.4%	Not measured
Low	68.5%	69.6%	
MMR status			
MMR-P	84.1%	84.2%	Not measured
MMR-D	15.9%	15.8%	

4.4.3 Tumoral MMP-9 expression

Expression of MMP-9 was quantified using the weight histoscore in the nucleus, cytoplasm and membrane. Stromal MMP-9 expression was also quantified, as this area is available when using full tissue sections for immunohistochemical analysis. None of the representative areas from the tumours studied expressed MMP-9 in the nucleus or membrane. Nine tumours (9.5%) were negative for cytoplasmic MMP-9 immunoreactivity and all stromal areas exhibited MMP-9 expression. Using the categorisation process described in section 4.2.2.2 cytoplasmic and stromal MMP-9 measurements were dichotomised around the median. Therefore, 46.3% of patients had high cytoplasmic MMP-9 and 50.5% of patients had high stromal MMP-9 expression.

4.4.3.1 Cytoplasmic MMP-9 expression

Figure 4.28 demonstrates that the expression of tumour cell cytoplasm MMP-9 does not precisely follow any data distributions (histogram, figure 4.32). The measurements ranged from 0.00 – 200.00 and had a median of 110.00 (IQR 0.00 – 230.0).

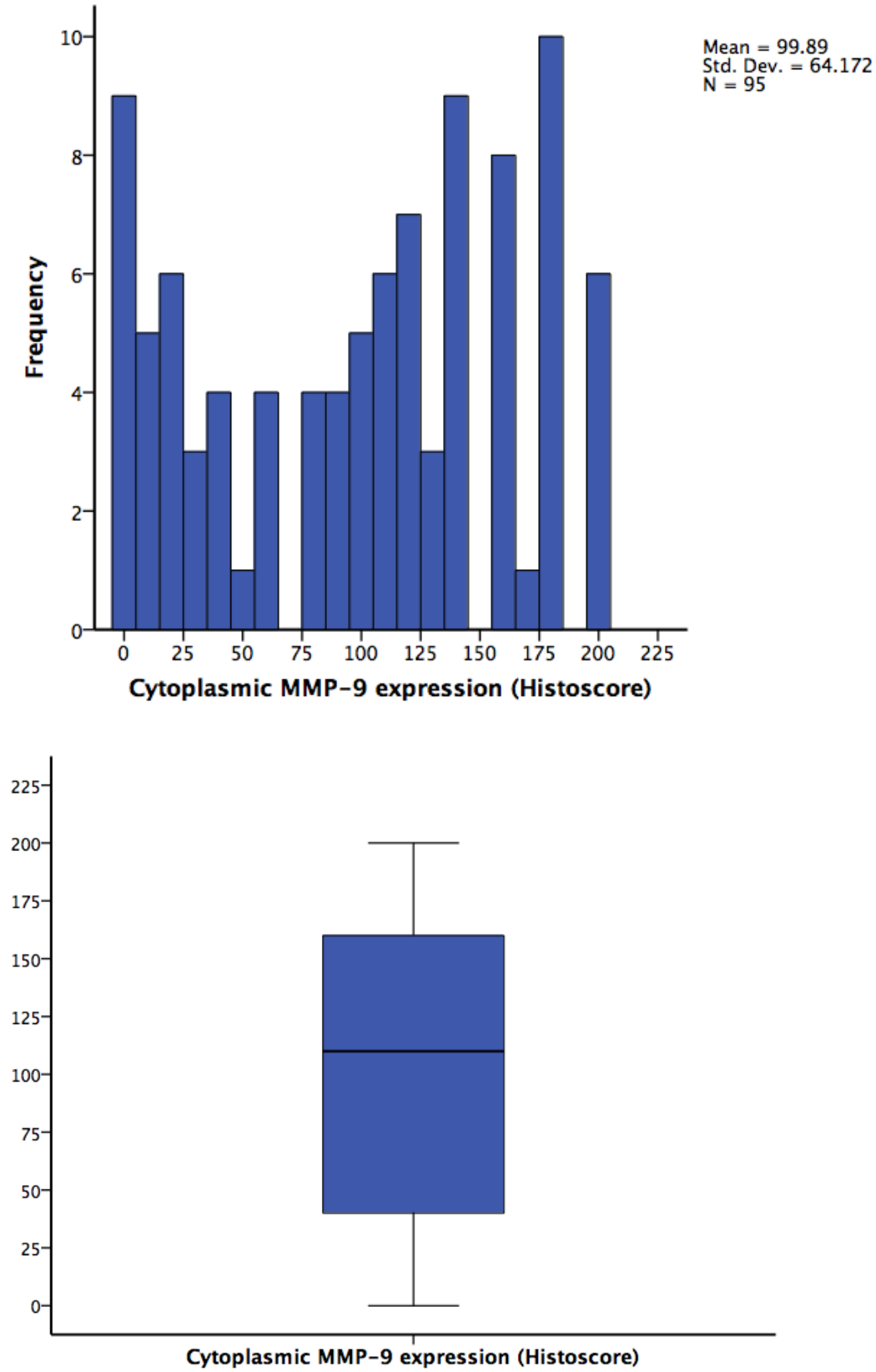
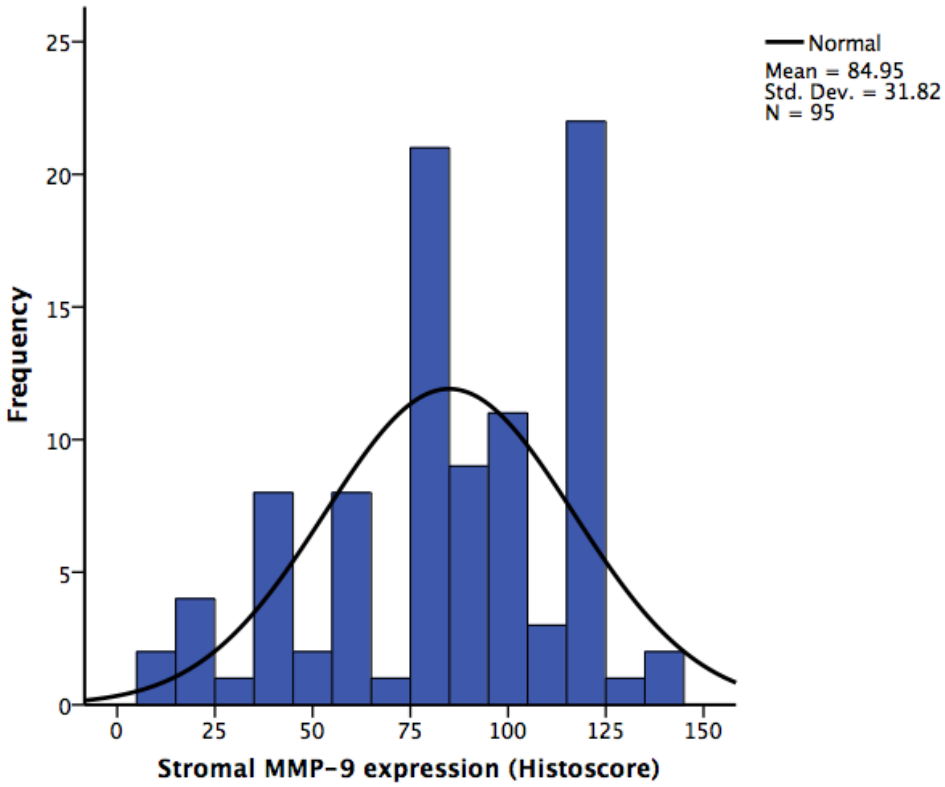


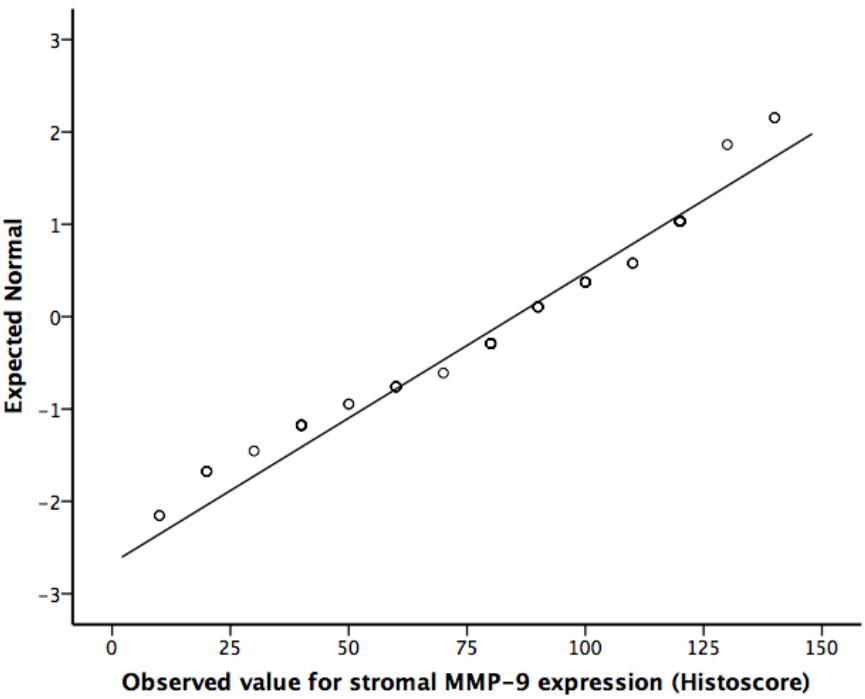
Figure 4.32: Distribution of measurements for cancer cell cytoplasmic MMP-9

Histogram demonstrating the distribution of cancer cell cytoplasmic MMP-9. Boxplot demonstrating the median measurement with interquartile range and outliers.

4.4.3.2 Stroma MMP-9 expression

Figure 4.29 demonstrates that the expression of stromal MMP-9 does not precisely follow a normal distribution (histogram, figure 4.33), which is supported by a Shapiro-Wilk statistic of 0.934, df 95, p<0.001. The measurements ranged from 10.00 – 140.00 and had a median of 90.00 (IQR 30.00 – 150.00).





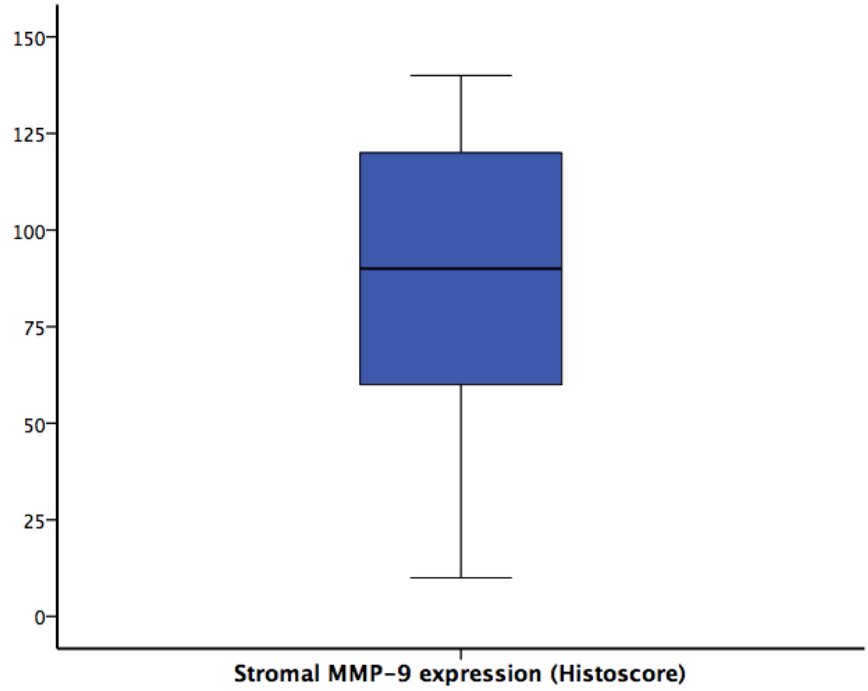


Figure 4.33: Distribution of measurements for cancer cell cytoplasmic MMP-9

Histogram demonstrating the distribution of cancer cell cytoplasmic MMP-9. Q-Q Plot of cancer cell cytoplasmic MMP-9 measurements. Boxplot demonstrating the median measurement with interquartile range and outliers.

4.4.4 Serum MMP-9 expression

Serum MMP-9 concentration in the context of health and disease is poorly understood. To help establish MMP-9 as a potential biomarker in colorectal cancer we quantified the concentrations of MMP-9 in normal healthy controls (n=30) and cancer patients (n=95) thus giving an overall sum of n=125.

4.4.4.1 Serum MMP-9 expression across all samples (n=125)

Figure 4.30 demonstrates that the expression of serum MMP-9 follows an exponential distribution (histogram, figure 4.34). The measurements ranged from 0.00 – 367.23 ng/ml and had a median of 76.15 ng/ml (IQR 0.00 – 203.49 ng/ml).

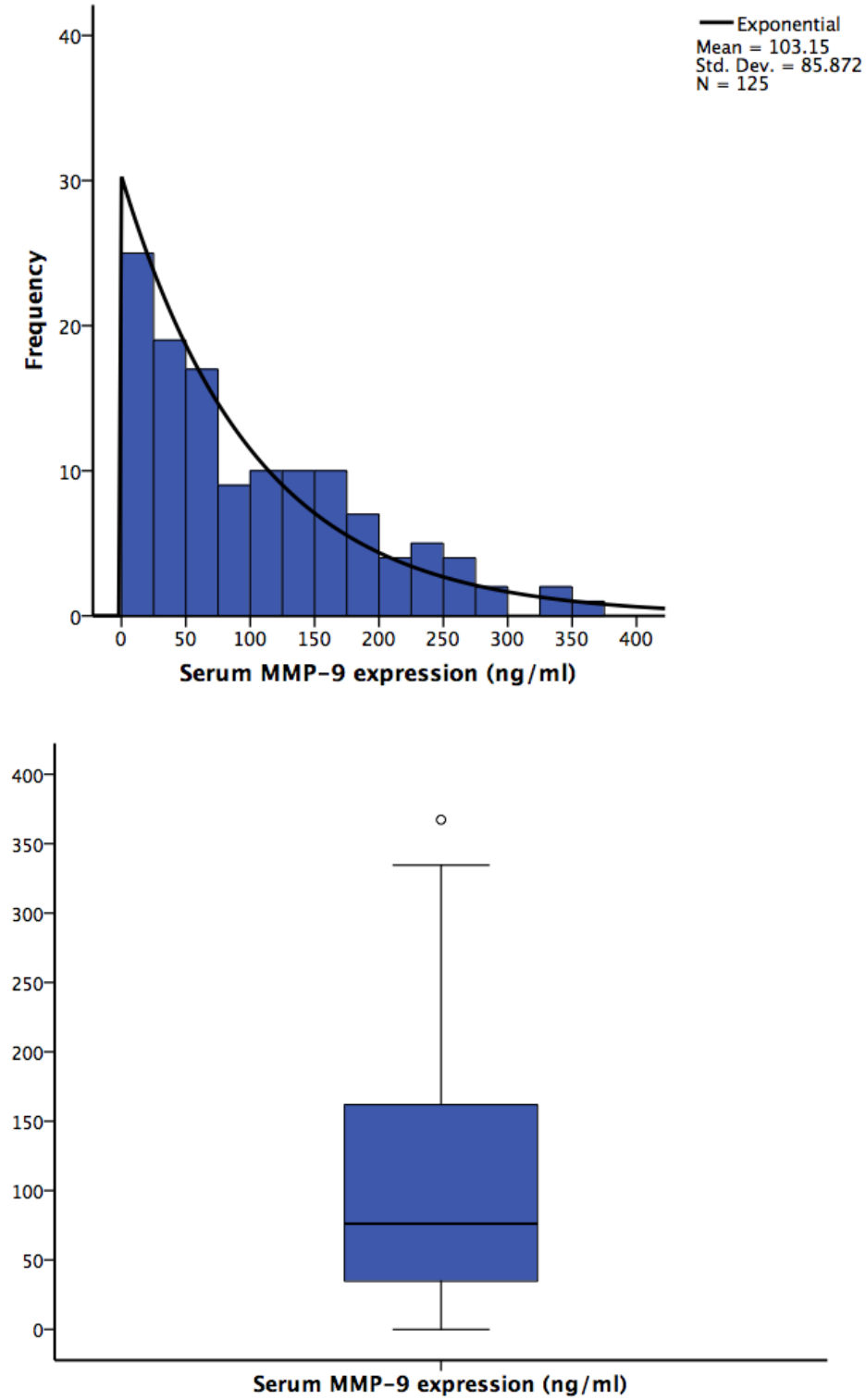


Figure 4.34: Distribution of measurements of serum MMP-9

Histogram demonstrating the distribution of serum MMP-9. Boxplot demonstrating the median measurement with interquartile range and outliers.

4.4.4.2 Serum MMP-9 expression stratified by healthy control or cancer patient status

There was no significant difference in the measurements of serum MMP-9 between patients and healthy controls (p=0.060, Mann-Whitney U, figure 4.35). The serum MMP-9 measurements in patients with colorectal cancer ranged from 0.00 – 367.23 ng/ml with a median of 65.25 ng/ml (IQR 0.00 – 175.00 ng/ml) compared with a range of 0.00 – 254.23 ng/ml and a median of 137.14 (IQR 0.82 – 273.46 ng/ml) in the healthy control group.

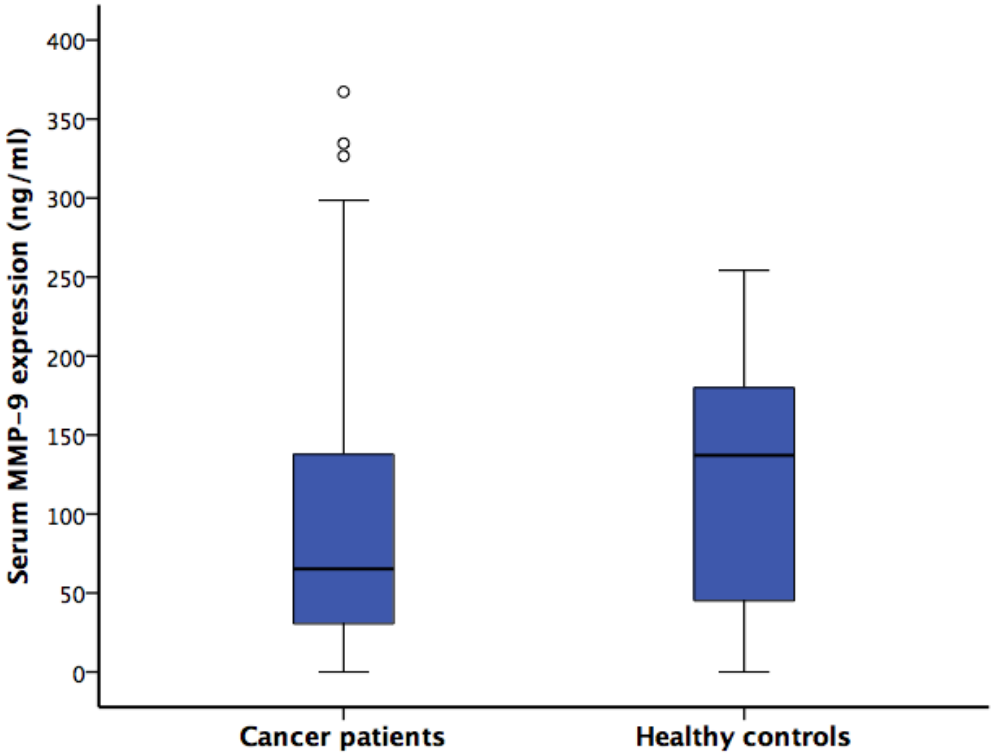


Figure 4.35: Serum MMP-9 concentration in patients and controls (p=0.060)

4.4.4.3 Serum MMP-9 expression in cancer patients

Figure 4.32 demonstrates that the expression of serum MMP-9 follows an exponential distribution (histogram, figure 4.36). The measurements ranged from 0.00 – 367.23 ng/ml and had a median of 65.25 ng/ml (IQR 0.00 – 175.10 ng/ml).

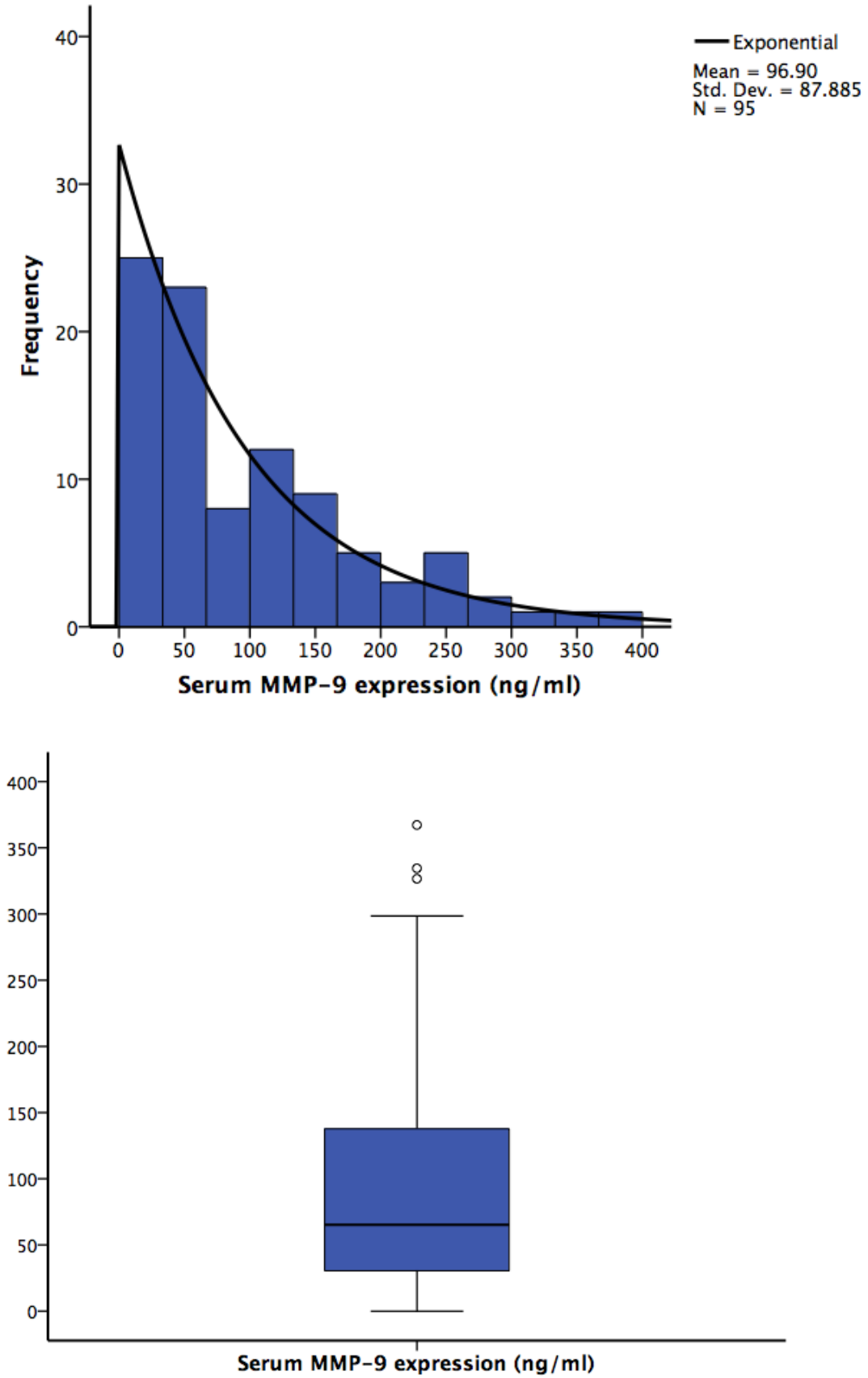


Figure 4.36: Distribution of measurements of serum MMP-9

Histogram demonstrating the distribution of serum MMP-9. Boxplot demonstrating the median measurement with interquartile range and outliers.

4.4.4.4 Serum MMP-9 survival prediction and cutoff choice

When ROC analysis was performed using recurrence as the endpoint, the AUC was calculated for serum MMP-9 as a continuous variable, and then categorised as a dichotomy around the median, tertiles and quartiles. The AUCs were 0.57 (95% CI 0.39 – 0.75, p=0.369) for serum MMP-9 as a continuous variable, 0.61 (95% CI 0.46 – 0.76, p=0.185) for serum MMP-9 as a median, 0.55 (95% CI 0.38 – 0.72, p=0.547) for serum MMP-9 as tertiles and an AUC of 0.57 (95% CI 0.39 – 0.75, p=0.397) for serum MMP-9 as quartiles. Furthermore, on review of the ROC curve, serum MMP-9 as a quartile closely follows that of continuous serum MMP-9 and therefore serum MMP-9 was dichotomised around the upper quartile which gave an AUC of 0.62 (95% CI 0.45 – 0.79, p=0.146) (figure 4.37).

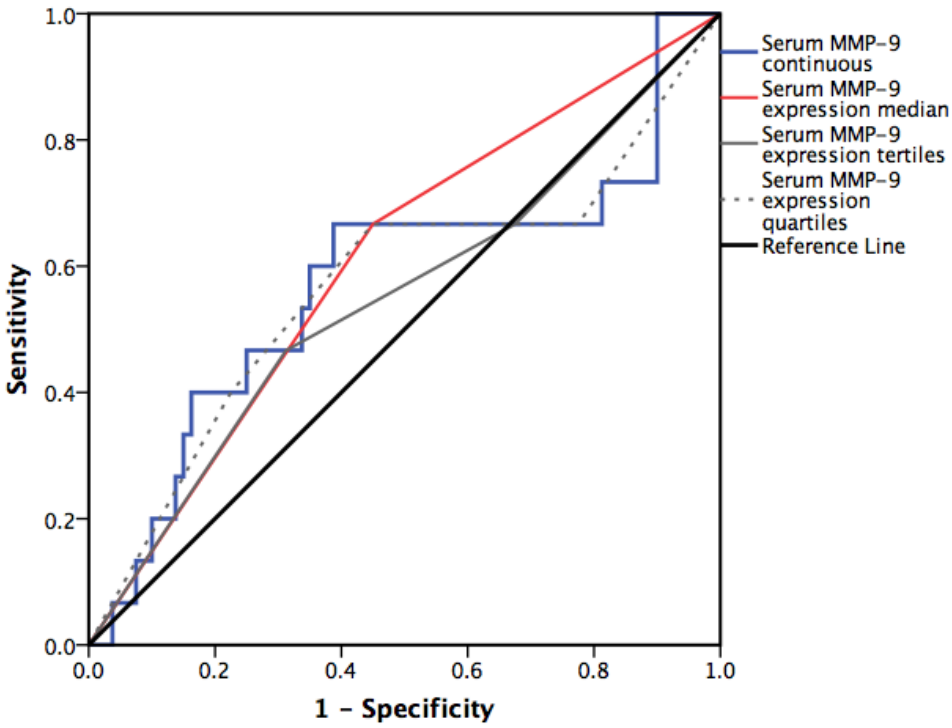


Figure 4.37: The predictive value of serum MMP-9 in identifying patients who will develop cancer recurrence

Receiver-operator-characteristic curve demonstrating the predictive value of serum MMP-9 in identifying patients who will develop cancer recurrence.

4.4.5 Cytoplasmic MMP-9 association with clinicopathological factors

4.4.5.1 Assessment of cytoplasmic MMP-9 as a continuous variable across different groups of categorical clinicopathological factors

None of the clinicopathological factors studied were associated with raised cytoplasmic MMP-9 expression. Gender (p=0.533, Mann-Whitney U), tumour site (p=0.121, Kruskal-Wallis), T-stage (p=0.604, Kruskal-Wallis), N-stage (p=0.869, Kruskal-Wallis), TNM stage (p=0.784, Kruskal-Wallis), tumour differentiation (p=0.186, Mann-Whitney U), categorical serum CRP (p=0.722, Mann-Whitney U) and categorical serum albumin (p=0.334, Mann-Whitney U) were not associated with continuous cytoplasmic MMP-9 expression.

4.4.5.2 Assessment of cytoplasmic MMP-9 as a continuous variable across different continuous data types of clinicopathological factors

On Spearman's rank test cytoplasmic MMP-9 was not associated with age (SCC 0.087, p=0.401), serum CRP (SCC 0.020, p=847) or serum albumin (SCC -0.182, p=0.078)

4.4.5.3 Assessment of cytoplasmic MMP-9 associations with clinicopathological factors: categorical data type

Raised cytoplasmic MMP-9 was not associated with any of the clinicopathological factors studied (table 4.5). Gender (p=0.821), tumour site (colon vs rectum) (p=0.203), tumour site (right vs left vs rectum) (p=0.081), tumour differentiation (p=0.621), T-stage (p=0.571), N-stage (p=0.868), TNM stage (p=0.921), serum CRP (p=0.844) and serum albumin (p=0.778) were not associated with categorical cytoplasmic MMP-9 expression (table 4.5).

Table 4.5: The relationship between tumour cytoplasmic MMP-9 expression and clinicopathological factors

Clinicopathological factors	Low cytoplasmic MMP-9 expression	High cytoplasmic MMP-9 expression	p-value
Sex			
Female	22 (43.1%)	20 (45.5%)	0.821
Male	29 (56.9%)	24 (54.5%)	
Tumour Site			
Colon	36 (70.6%)	36 (81.8%)	0.203
Rectum	15 (29.4%)	8 (18.2%)	
Tumour Site			
Right	26 (51.0%)	20 (45.5%)	0.081
Left	10 (19.6%)	17 (38.6%)	
Rectum	15 (29.4%)	7 (15.9%)	
Differentiation			
Well-Mod	48 (94.1%)	43 (97.7%)	0.621
Poor	3 (5.9%)	1 (2.3%)	
T stage			
1	5 (9.8%)	2 (4.5%)	0.571
2	7 (13.7%)	9 (20.5%)	
3	30 (58.8%)	23 (52.3%)	
4	9 (17.6%)	10 (22.7%)	
N stage			
0	34 (66.7%)	29 (65.9%)	0.868
1	11 (21.6%)	11 (25.0%)	
2	6 (11.8%)	4 (9.1%)	
TNM stage (simplified)			
I	9 (17.6%)	9 (20.0%)	0.921
II	25 (49.0%)	20 (45.5%)	
III	17 (33.3%)	15 (34.1%)	
Serum CRP			
Normal	38 (74.5%)	32 (72.7%)	0.844
High	13 (25.5%)	12 (27.3%)	
Serum albumin			
Normal	35 (68.6%)	29 (65.9%)	0.778
Low	16 (31.4%)	15 (34.1%)	

Bonferroni adjustment = <0.0056
* Fishers exact test

4.4.6 Stromal MMP-9 association with clinicopathological factors

4.4.6.1 Assessment of stromal MMP-9 as a continuous variable across different groups of categorical clinicopathological factors

Only female gender (p=0.036, Mann-Whitney U) showed a trend towards an association with higher stromal MMP-9 expression, however, this was not significant following Bonferroni adjustment (p<0.0056). Tumour site (p=0.353, Kruskal-Wallis), tumour differentiation (p=0.865, Mann-Whitney U), T-stage (p=0.383, Kruskal-Wallis), N-stage

(p=0.981, Kruskal-Wallis), TNM stage (p=0.797, Kruskal-Wallis), categorical serum CRP (p=0.644, Mann-Whitney U) and categorical serum albumin (p=0.059, Mann-Whitney U) were not associated with continuous stromal MMP-9 expression.

4.4.6.2 Assessment of stromal MMP-9 as a continuous variable across different continuous data types of clinicopathological factors

On Spearman's rank test stromal MMP-9 was not associated with age (SCC -0.098, p=0.347), serum CRP (SCC 0.034, p=0.744) or serum albumin (SCC -0.175, p=0.090)

4.4.6.3 Assessment of stromal MMP-9 associations with clinicopathological factors: categorical data type

Raised stromal MMP-9 was not associated with any of the clinicopathological factors studied (table 4.6). Gender (p=0.118), tumour site (colon vs rectum) (p=0.209), tumour site (right vs left vs rectum) (p=0.464), tumour differentiation (p=0.983), T-stage (p=0.660), N-stage (p=0.855), TNM stage (p=0.854), serum CRP (p=0.526) and serum albumin (p=0.144) were not associated with categorical stromal MMP-9 expression (table 4.6)

Table 4.6: The relationship between tumour stromal MMP-9 expression and clinicopathological factors

Clinicopathological factors	Low stromal MMP-9 expression	High stromal MMP-9 expression	p-value
Sex			
Female	17 (36.2%)	25 (52.1%)	0.118
Male	30 (63.8%)	23 (47.9%)	
Tumour Site			
Colon	33 (70.2%)	39 (81.2%)	0.209
Rectum	14 (29.8%)	9 (18.8%)	
Tumour Site			
Right	20 (42.6%)	26 (54.2%)	0.464
Left	14 (29.8%)	13 (27.1%)	
Rectum	13 (27.7%)	9 (18.8%)	
Differentiation			
Well-Mod	45 (95.7%)	46 (95.8%)	0.983
Poor	2 (4.3%)	2 (4.2%)	
T stage			
1	5 (10.6%)	2 (4.2%)	0.660
2	7 (14.9%)	9 (18.8%)	
3	26 (55.3%)	27 (56.2%)	
4	9 (19.1%)	10 (20.8%)	
N stage			
0	30 (63.8%)	33 (68.8%)	0.855
1	12 (25.5%)	10 (20.8%)	
2	5 (10.6%)	5 (10.4%)	
TNM stage (simplified)			
I	9 (19.1%)	9 (18.8%)	0.854
II	21 (44.7%)	24 (50.0%)	
III	17 (36.2%)	15 (31.2%)	
Serum CRP			
Normal	36 (76.6%)	34 (70.8%)	0.526
High	11 (23.4%)	14 (29.2%)	
Serum albumin			
Normal	35 (74.5%)	29 (60.4%)	0.144
Low	12 (25.5%)	19 (39.6%)	

Bonferroni adjustment = <0.0056
* Fishers exact test

4.4.7 Serum MMP-9 association with clinicopathological factors

4.4.7.1 Assessment of serum MMP-9 as a continuous variable across different groups of categorical clinicopathological factors

Only poor tumour differentiation (p=0.039, Mann-Whitney U) showed a trend towards an association with higher serum MMP-9 expression, however, this was not significant following Bonferroni adjustment (p<0.0056). Gender (p=0.633, Mann-Whitney U), tumour site (p=0.193, Kruskal-Wallis), tumour differentiation (p=0.039, Mann-Whitney U), T-stage (p=0.849, Kruskal-Wallis), N-stage (p=0.718, Kruskal-Wallis), TNM stage (p=0.610,

Kruskal-Wallis) categorical serum CRP (p=0.548, Mann-Whitney U), categorical serum albumin (p=0.162, Mann-Whitney U) were not associated with continuous serum MMP-9 expression.

4.4.7.2 Assessment of serum MMP-9 as a continuous variable across different continuous data types of clinicopathological factors

On Spearman's rank test serum MMP-9 was not associated with Age (SCC -0.083, p=0.714), serum CRP (SCC -0.001, p=0.996) or serum albumin (SCC -0.095, p=0.360)

4.4.7.3 Assessment of serum MMP-9 associations with clinicopathological factors: categorical data type

Raised serum MMP-9 was not associated with any of the clinicopathological factors studied following Bonferroni adjustment (p<0.0056). Gender (p=0.470), tumour site (colon vs rectum) (p=0.719), tumour site (right vs left vs rectum) (p=0.695), tumour differentiation (p=0.307), T-stage (p=0.025), N-stage (p=0.247), TNM stage (p=0.749), serum CRP (p=0.244) and serum albumin (p=0.512) were not associated with categorical serum MMP-9 expression (table 4.7).

Table 4.7: The relationship between tumour serum MMP-9 expression and clinicopathological factors

Clinicopathological factors	Low serum MMP-9 expression	High serum MMP-9 expression	p-value
Sex			
Female	35 (46.1%)	7 (36.8%)	0.470
Male	41 (53.9%)	12 (63.2%)	
Tumour Site			
Colon	57 (75.0%)	15 (78.9%)	0.719
Rectum	19 (25.0%)	4 (21.1%)	
Tumour Site			
Right	36 (47.4&)	10 (52.6%)	0.695
Left	21 (27.6%)	6 (31.6%)	
Rectum	19 (25.0%)	3 (15.8%)	
Differentiation			
Well-Mod	72 (94.7%)	19 (100.0%)	0.307
Poor	4 (5.3%)	0 (0.0%)	
T stage			
1	7 (9.2%)	0 (0.0%)	0.025
2	12 (15.8%)	4 (21.1%)	
3	46 (60.5%)	7 (36.8%)	
4	11 (14.5%)	8 (42.1%)	
N stage			
0	49 (64.5%)	14 (73.7%)	0.247
1	17 (22.4%)	5 (26.3%)	
2	10 (13.2%)	0 (0.0%)	
TNM stage (simplified)			
I	14 (18.4%)	4 (21.1%)	0.749
II	35 (46.1%)	10 (52.6%)	
III	27 (35.5%)	5 (26.3%)	
Serum CRP			
Normal	58 (76.3%)	12 (63.2%)	0.244
High	18 (23.7%)	7 (36.8%)	
Serum albumin			
Normal	50 (65.8%)	14 (73.7%)	0.512
Low	26 (34.2%)	5 (26.3%)	

Bonferroni adjustment = <0.0056

* Fishers exact test

4.4.8 MMP-9 interrelationships

To adjust for multiple correlations we used the Bonferroni method for adjusting alpha for multiple comparisons. For correlations to be significant a p-value must be less than $p<0.0167$ and a Spearman's correlation coefficient greater than 0.300 or less than -0.300. Serum MMP-9 was associated with higher stomal MMP-9 expression (SCC 0.319, $p=0.002$, figure 4.38) and cytoplasmic MMP-9 expression (SCC 0.393, $p<0.001$, figure 4.39) (table 4.8). Stromal MMP-9 correlated with higher cytoplasmic MMP-9 expression levels of cytoplasmic MMP-9 (SCC 0.699, $p<0.001$, figure 4.40) (table 4.8).

Table 4.8: Spearman correlation coefficients for the assessment of interrelationships between serum and tumour MMP-9 expression

		Stromal MMP-9	**Cytoplasmic MMP-9**
Serum MMP-9	SCC	0.319	0.393
	P-value	0.002	<0.001
Stromal MMP-9	SCC		0.684
	P-value		<0.001

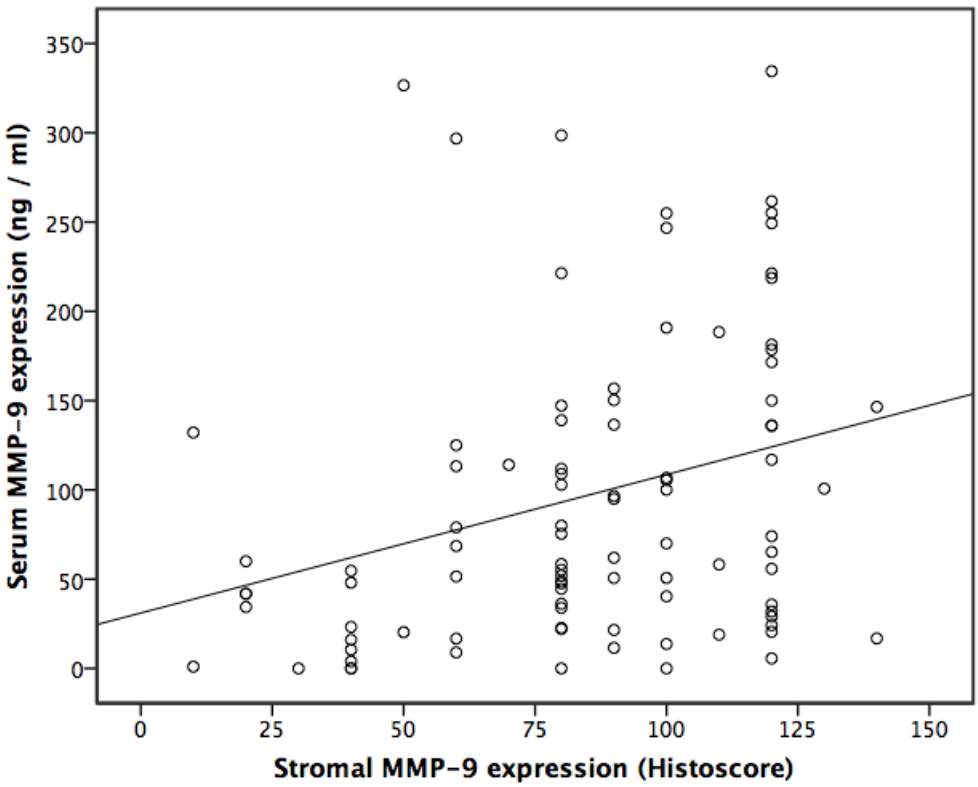


Figure 4.38: Correlation between serum MMP-9 and stromal MMP-9 expression (p=0.002, SCC 0.319)

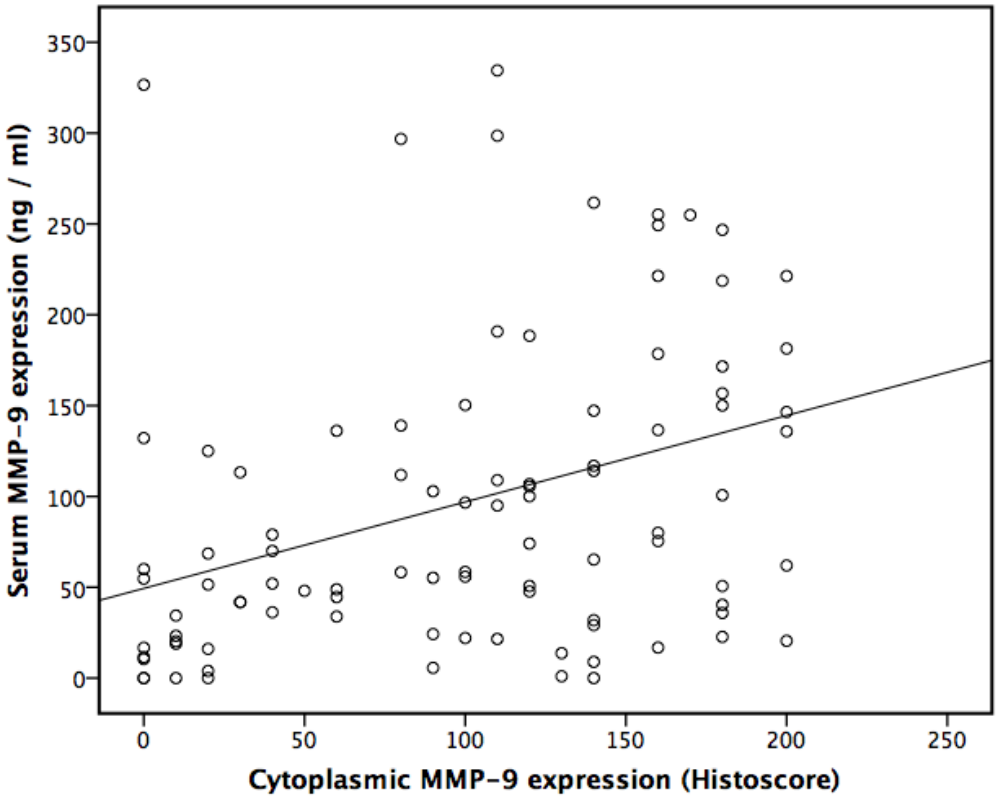


Figure 4.39: Correlation between serum MMP-9 and cytoplasmic MMP-9 expression ($p<0.001$, SCC 0.393)

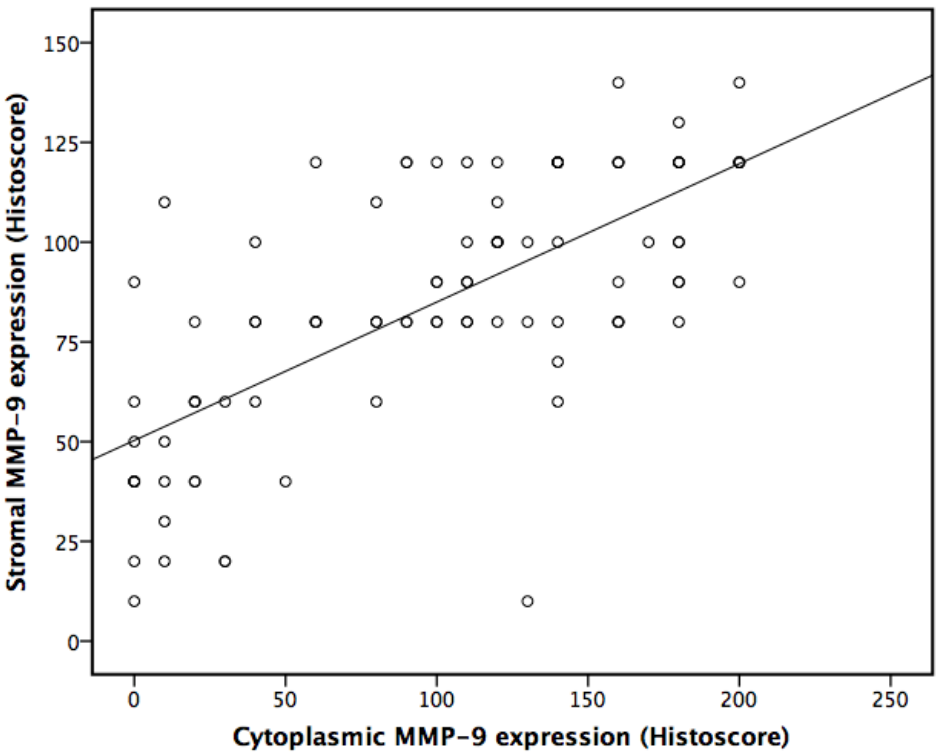


Figure 4.40: Correlation between Cytoplasmic MMP-9 and stromal MMP-9 expression ($p<0.001$, SCC 0.686)

4.4.9 Association of MMP-9 expression, clinicopathological factors and survival

During follow-up there were 15 (22.2%) recurrences and 20 (38.4%) deaths.

4.4.9.1 *Univariable recurrence-free survival - cytoplasmic MMP-9 expression*

Tumour cytoplasmic MMP-9 measurements were not significantly different in patients who went on to develop disease recurrence (p=0.263, Mann-Whitney U, figure 4.41). The median for patients with recurrence was 120.00 (IQR 60.00 – 180.00) compared with 100.00 (IQR 0.00 – 225.00) in the non-recurrence group.

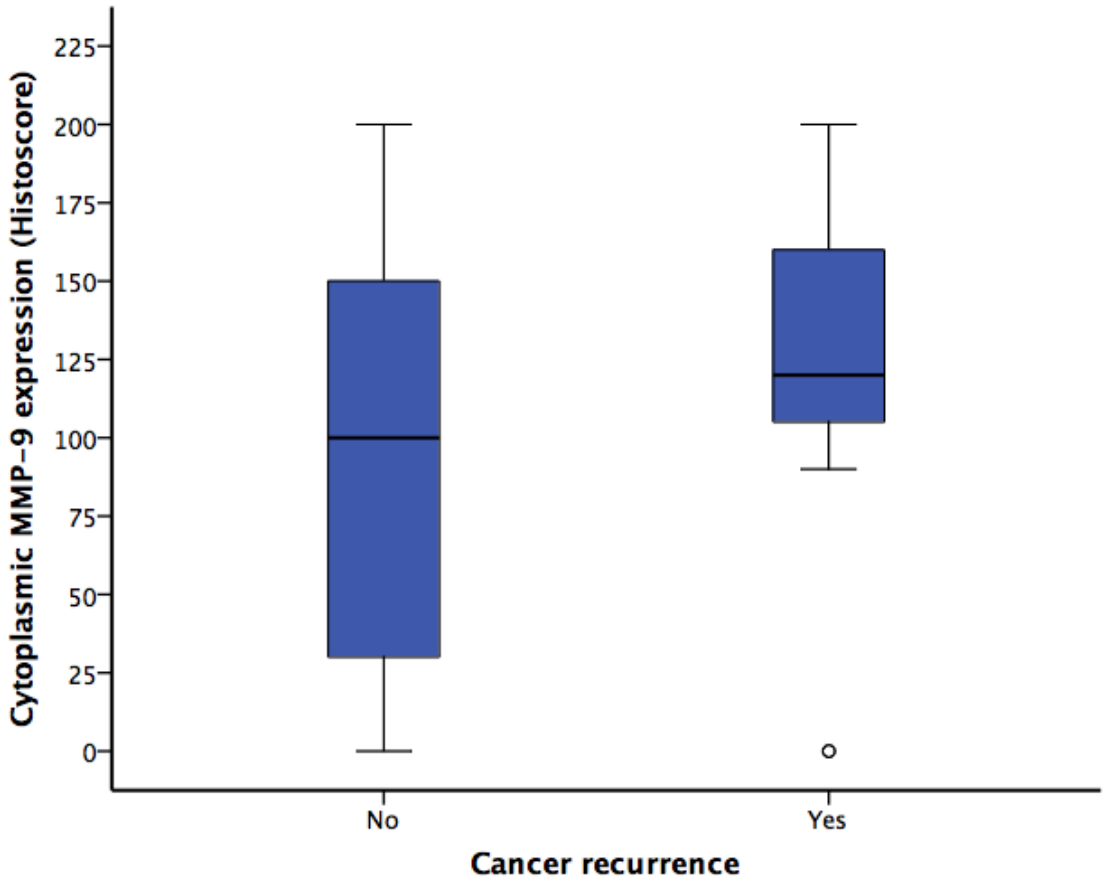
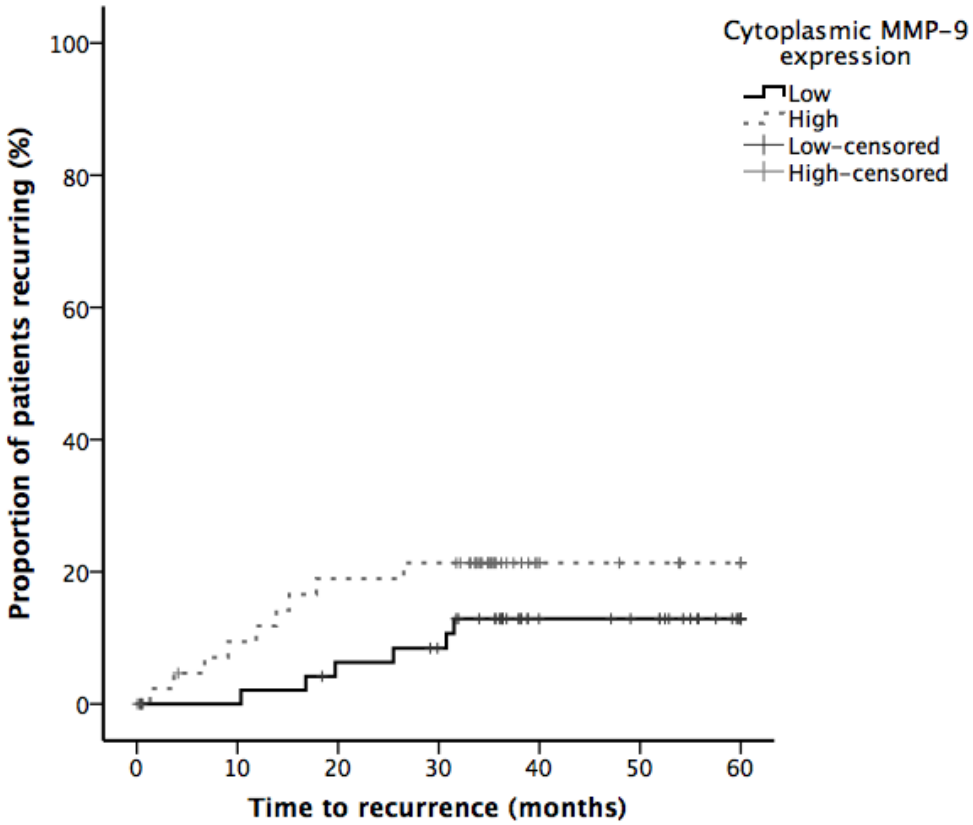


Figure 4.41: The distribution of cytoplasmic MMP-9 measurements in patients with and without disease recurrence (p=0.263)

On logrank analysis, raised cytoplasmic MMP-9 was not significantly associated with poor recurrence-free survival (p=0.226, figure 4.42). The mean survival for patients with a raised cytoplasmic MMP-9 was 49.7 months (95% CI 43.7 – 55.8) compared with 55.1 months (95% CI 51.5 – 58.8) in the low cytoplasmic MMP-9 group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	51	48	44	41	22	20	8
High	44	38	34	33	8	6	4

Figure 4.42: The relationship between cytoplasmic MMP-9 expression and recurrence-free survival in patients with colorectal cancer (p=0.226)

Kaplan-Meier curve demonstrating the proportion of patients recurring stratified by cytoplasmic MMP-9 *expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.*

On Cox univariable regression analysis raised cytoplasmic MMP-9 was not significantly associated with poor recurrence-free survival when dichotomised as a categorical variable (HR 1.87 (95% CI 0.67 – 5.27), p=0.233) or as a continuous variable (HR 1.01 (95% CI 1.00 – 1.01), p=0.191). In addition to the non-significant associations observed between cytoplasmic MMP-9 expression and disease recurrence, its predictive value was poor. When ROC analysis was performed using recurrence as the endpoint, the AUC was 0.59 (95% CI 0.44 – 0.74, p=0.264) for cytoplasmic MMP-9 as a continuous variable and an AUC of 0.58 (95% CI 0.42 – 0.74, p=0.320) for cytoplasmic MMP-9 as a categorical variable (figure 4.43).

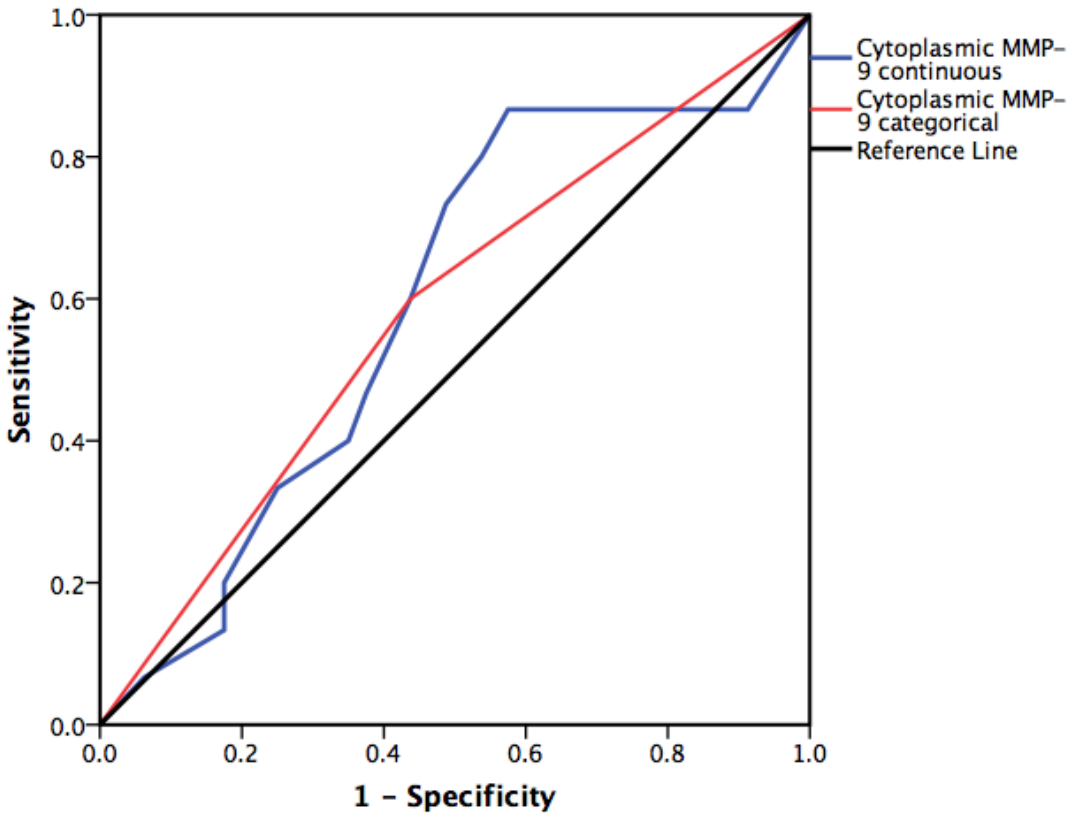


Figure 4.43: The predictive value of cytoplasmic MMP-9 in identifying patients who will develop cancer recurrence

Receiver-operator-characteristic curve demonstrating the predictive value of cytoplasmic MMP-9 *in identifying patients who will develop disease recurrence.*

4.4.9.2 Univariable recurrence-free survival - stromal MMP-9 expression

There was no statistically significant difference between stromal MMP-9 measurements of patients who did and did not develop cancer recurrence during follow-up (p=0.717, Mann-Whitney U, figure 4.44). The median stromal MMP-9 measurement was 80.00 (IQR 60.00 – 100.00) in the recurrence group compared to 80.00 (IQR 20.00 – 140.00) in the non-recurrence group.

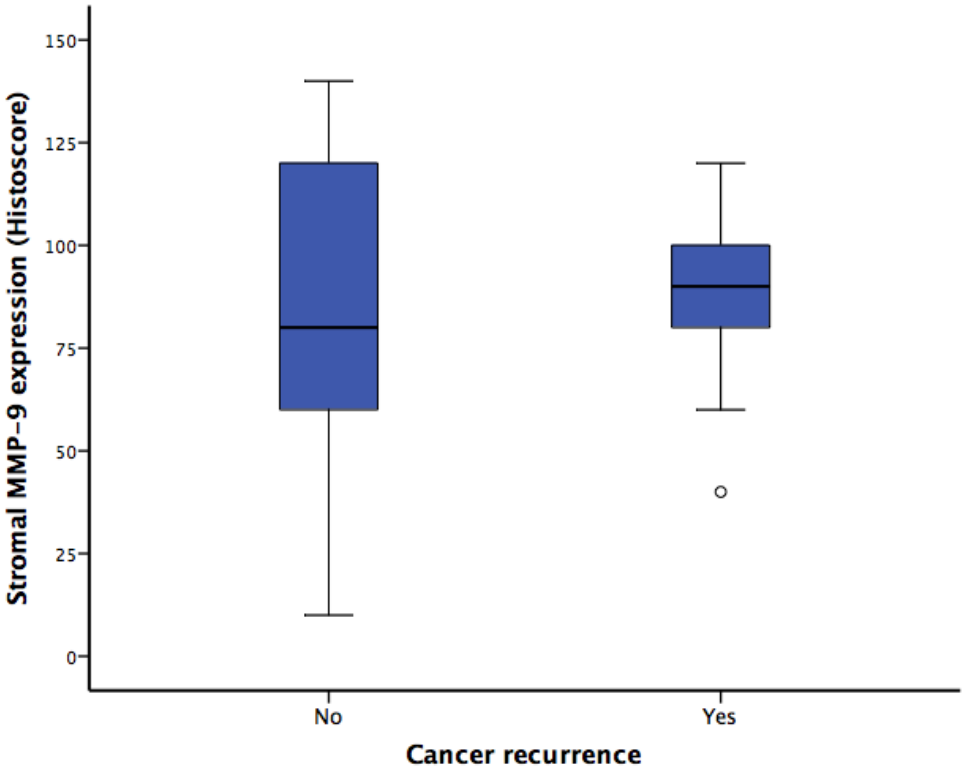
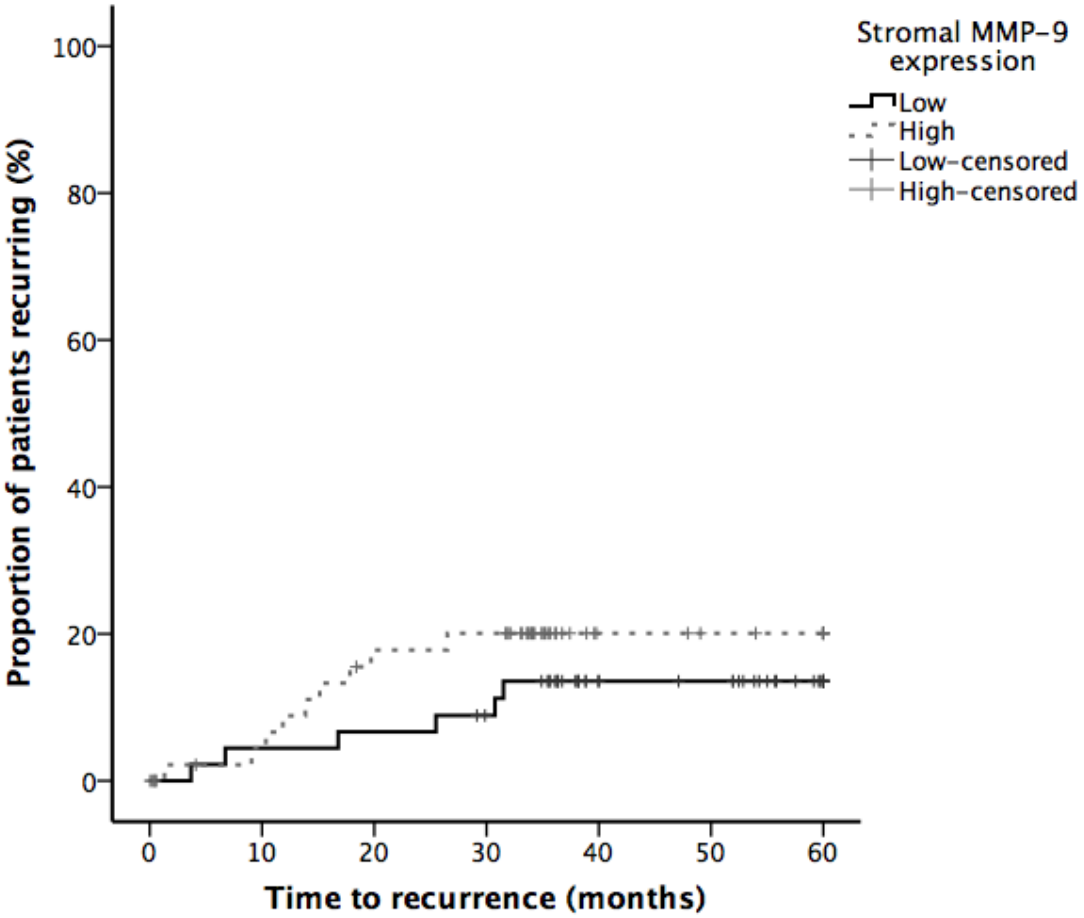


Figure 4.44: The distribution of stromal MMP-9 measurements in patients with and without cancer recurrence (p=0.717)

On logrank analysis raised stromal MMP-9 was not associated with poor recurrence-free survival (p=0.375, figure 4.45). The mean survival for patients with raised stromal MMP-9 was 50.8 months (95% CI 45.3 – 56.2) compared with 54.5 months (95% CI 50.2 – 58.7) in the low stromal MMP-9 group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Low	47	43	42	39	21	19	6
High	48	43	36	35	9	7	6

Figure 4.45: The relationship between stromal MMP-9 expression and recurrence-free survival (p=0.375)

Kaplan-Meier curve demonstrating the proportion of patients recurring stratified by stromal MMP-9 *expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.*

On Cox univariable regression analysis stromal MMP-9 was not associated with poor recurrence free survival when dichotomised as a categorical variable (HR 1.59 (95% CI 0.57 – 4.47), p=0.379) or as a continuous variable (HR 1.01 (95% CI 1.00 – 1.02), p=0.524). In addition to the non-significant associations observed between stromal MMP-9 and disease recurrence, its predictive value is relatively poor. When ROC analysis was performed using recurrence as the endpoint, the AUC was 0.53 (95% CI 0.40 – 0.66, p=0.721) for stromal MMP-9 as a continuous variable and an AUC of 0.56 (95% CI 0.40 – 0.71, p=0.491) for stromal MMP-9 as a categorical variable (figure 4.46).

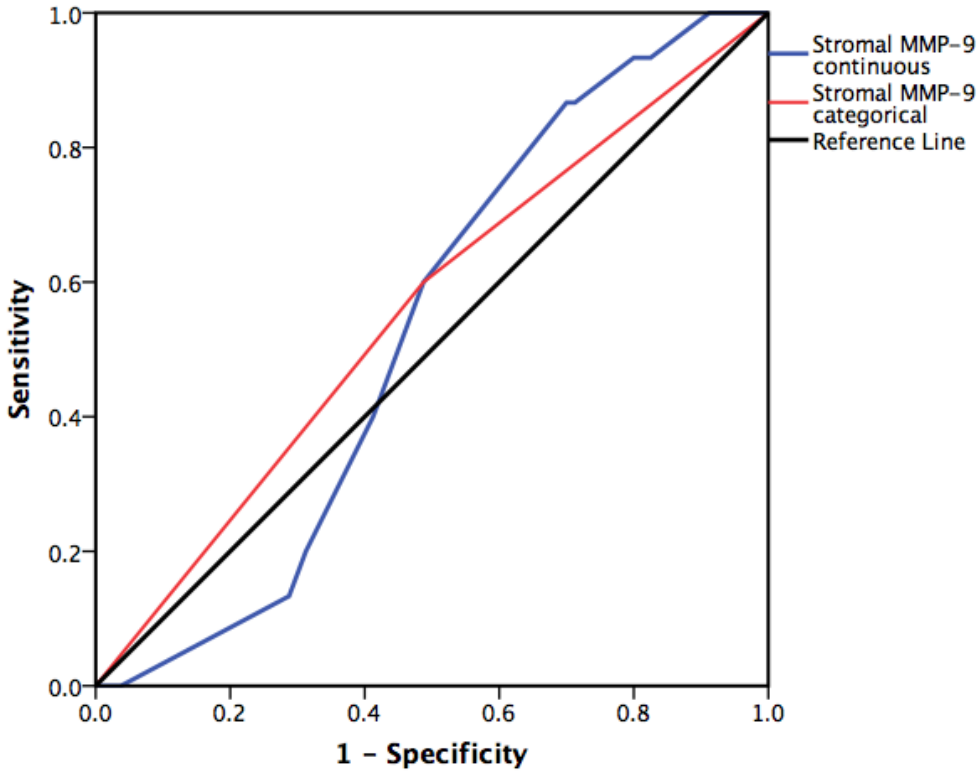


Figure 4.46: Predictive value of stromal MMP-9 in identifying patients who will develop cancer recurrence

Receiver-operator-characteristic curve demonstrating the predictive value of stromal MMP-9 *in identifying patients who will develop cancer recurrence during follow-up.*

4.4.9.3 Univariable recurrence-free survival - serum MMP-9 expression

Serum MMP-9 measurements were higher in patients who went on to develop cancer recurrence (p=0.369, Mann-Whitney U, figure 4.47). The median for patients with recurrence was 106.80 ng/ml (IQR 0.00 – 281.52 ng/ml) compared with 59.23 ng/ml (IQR 0.00 – 161.99 ng/ml) in the non-recurrence group.

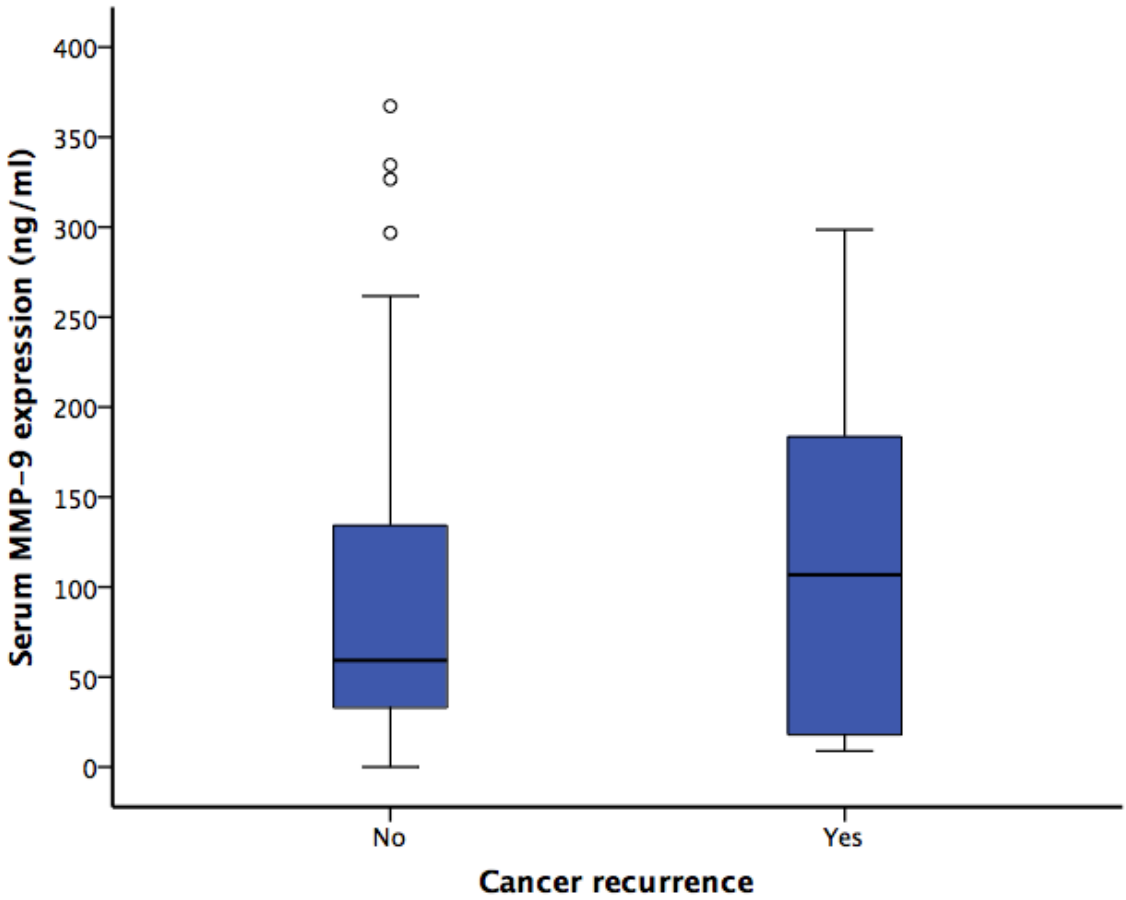
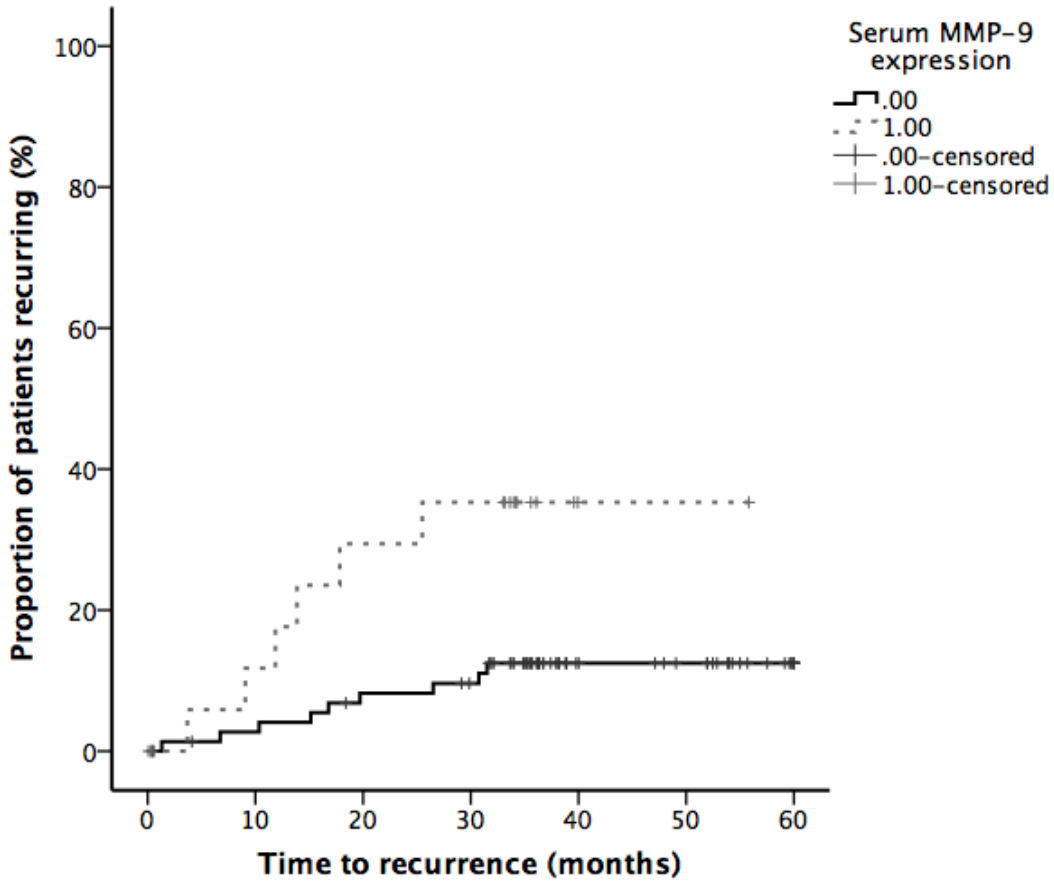


Figure 4.47: The distribution of serum MMP-9 measurements in patients with and without disease recurrence (p=0.369)

On logrank analysis, raised serum MMP-9 was significantly associated with poor recurrence free survival (p=0.014, figure 4.48). The mean survival for patients with a raised serum MMP-9 was 40.9 months (95% CI 31.1 – 50.7) compared with 54.7 months (95% CI 51.4 – 58.0) in the low serum MMP-9 group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	76	71	66	63	29	24	12
High	19	15	12	11	1	1	0

Figure 4.48: The relationship between serum MMP-9 expression and recurrence-free survival in patients with colorectal cancer (p=0.014)

Kaplan-Meier curve demonstrating the proportion of patients recurring stratified by serum MMP-9 *expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.*

On Cox univariable regression analysis raised serum MMP-9 was significantly associated with poor recurrence free survival when dichotomised as a categorical variable (HR 3.37 (95% CI 1.20 – 9.48), p=0.021) but not as a continuous variable (HR 1.00 (95% CI 1.00 – 1.01), p=0.117). Despite the significant associations observed between serum MMP-9 and disease recurrence, its predictive value remains poor. When ROC analysis was performed using recurrence as the endpoint, the AUC was 0.62 (95% CI 0.45 – 0.79, p=0.146) for serum MMP-9 as a categorical variable.

4.4.9.4 Univariable Overall survival - cytoplasmic MMP-9 expression

Cytoplasmic MMP-9 measurements were not statistically higher in patients who died during the follow up period (p=0.445, Mann-Whitney U, figure 4.49). The median measurement for patients who died was 120.00 (IQR 47.50 – 192.50) compared with 100.00 (IQR 0.00 – 230.00) in the alive group.

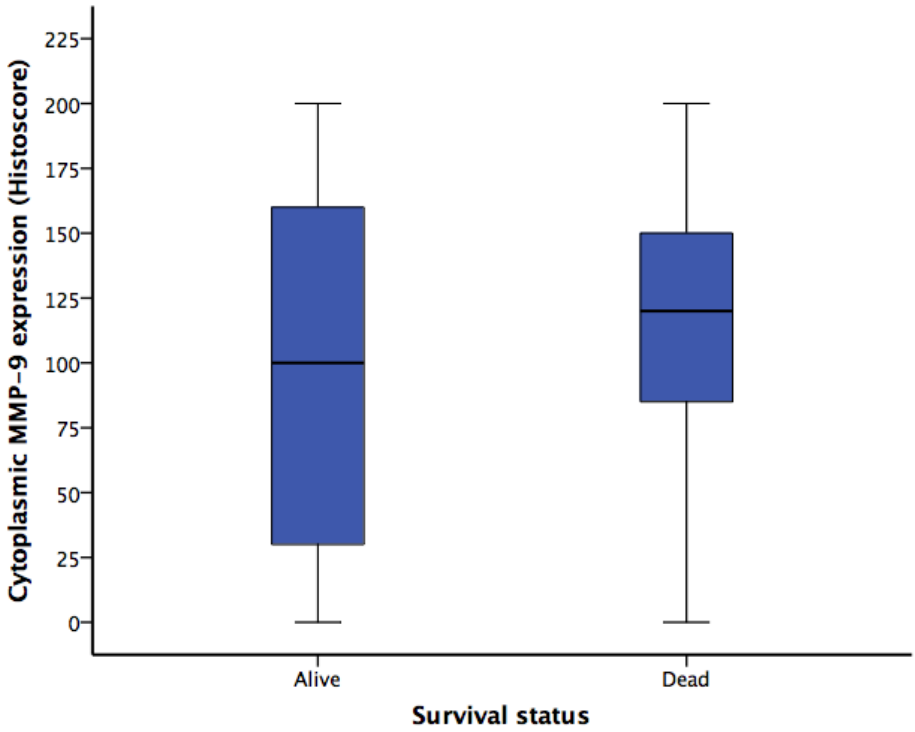
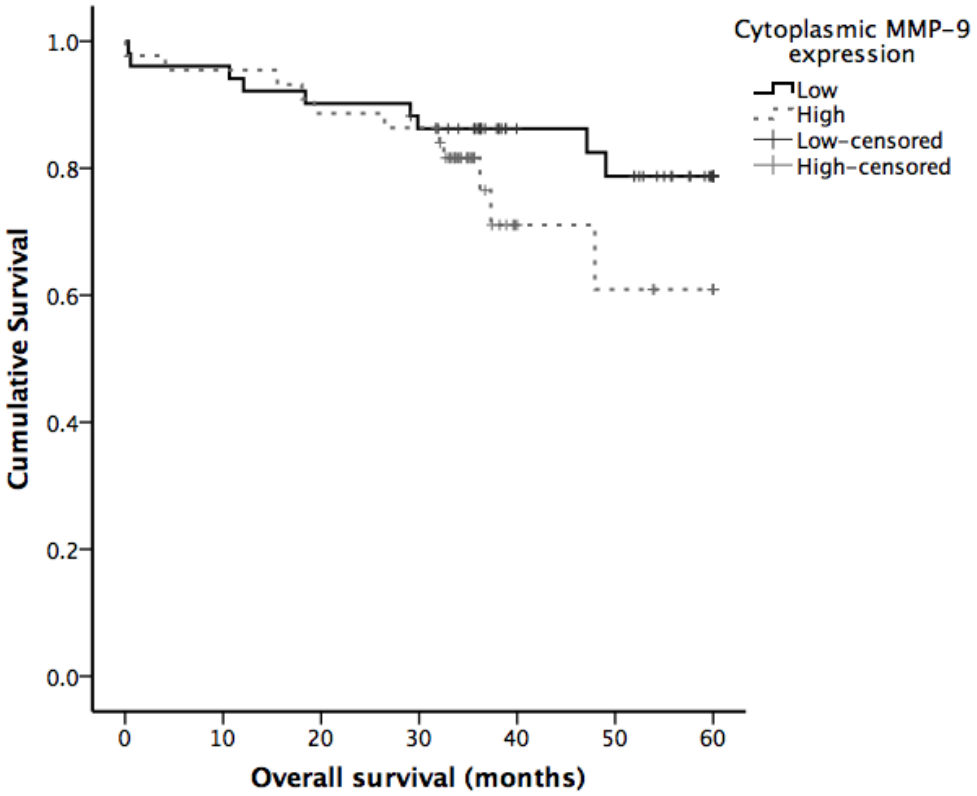


Figure 4.49: The distribution of cytoplasmic MMP-9 measurements in patients stratified by survival status (p=0.445)

On logrank analysis, raised cytoplasmic MMP-9 expression was not significantly associated with poor overall survival (p=0.209, figure 4.50). The mean survival for patients with a raised cytoplasmic MMP-9 expression was 48.7 months (95% CI 42.9 – 54.5) compared with 52.8 months (95% CI 48.3 – 57.4) in the low cytoplasmic MMP-9 expression group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Low	51	49	46	43	23	21	8
High	44	42	39	38	8	6	4

Figure 4.50: The relationship between cytoplasmic MMP-9 expression and overall survival in patients with colorectal cancer (p=0.209)

Kaplan-Meier curve demonstrating the proportion of patients surviving stratified by cytoplasmic MMP-9 *expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.*

On Cox univariable regression analysis raised cytoplasmic MMP-9 was not significantly associated with poor overall survival when dichotomised as a categorical variable (HR 1.77 (95% CI 0.72 – 4.34), p=0.215) or when analysed as a continuous variable (HR 1.01 95% CI 1.00 – 1.01, p=0.157). In addition to the non-significant associations with survival, cytoplasmic MMP-9 did not predict death during the follow up period as a categorical variable (AUC of 0.56 (95% CI 0.43 – 0.68, p=0.446), ROC analysis) or continuous variable (AUC of 0.56 (95% CI 0.41 – 0.70, p=0.451), ROC analysis), (figure 4.51).

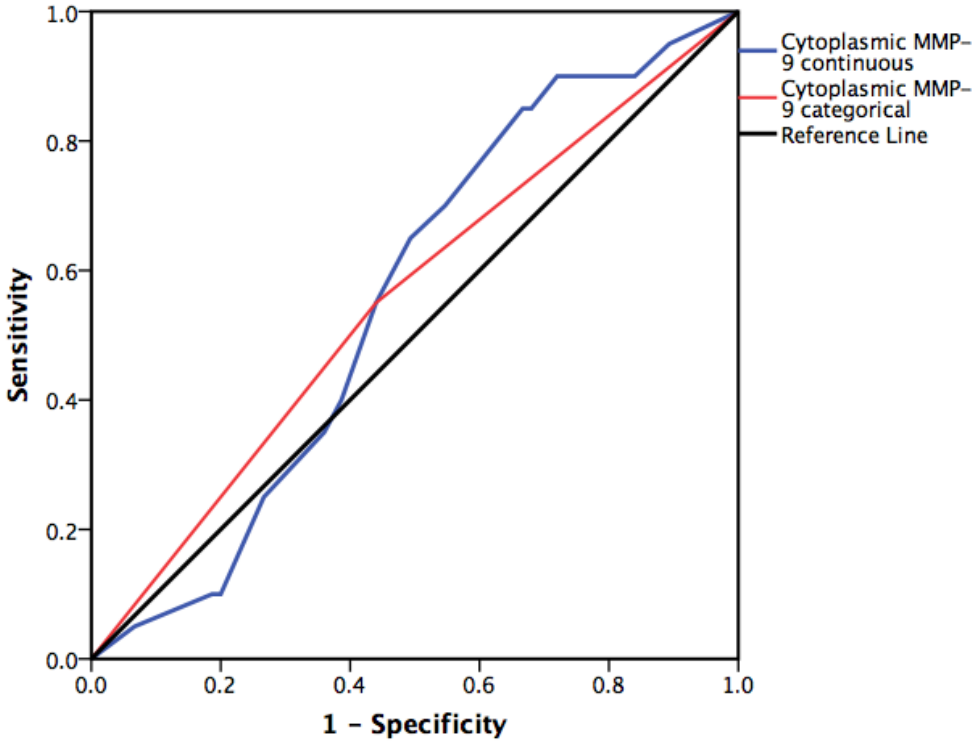


Figure 4.51: Predictive value of cytoplasmic MMP-9 in identifying patients who will die during follow-up

Receiver-operator-characteristic curve demonstrating the predictive value of cytoplasmic MMP-9 *in identifying patients who will die during follow-up.*

4.4.9.5 Univariable overall survival - stromal MMP-9 expression

Stromal MMP-9 measurements were not significantly different in patients who died during the follow up period (p=0.114, Mann-Whitney U, figure 4.52). The median stromal MMP-9 was 80.00 (IQR 20.00 – 140.00) in the alive group compared to 95.00 (IQR 55.00 – 135.00) in patients who died during follow-up.

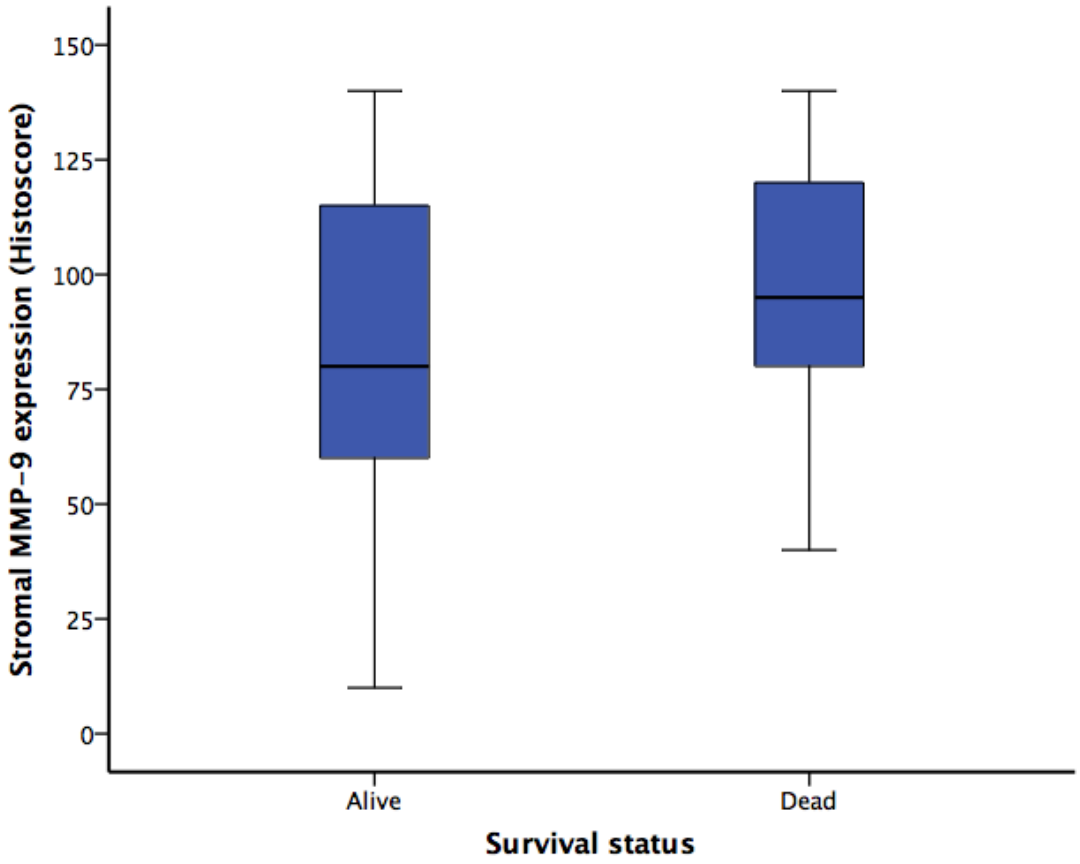
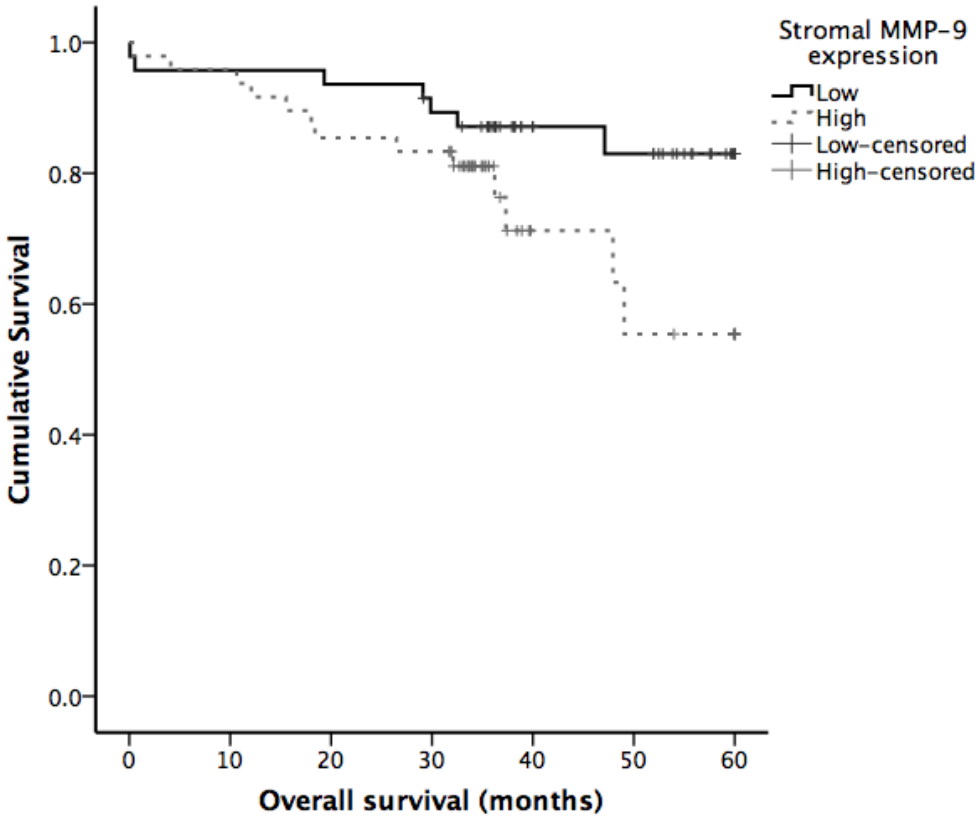


Figure 4.52: The distribution of stromal MMP-9 measurements in patients stratified by survival status (p=0.114)

On logrank analysis stromal MMP-9 was not associated with poor overall survival (p=0.064, figure 4.53). The mean survival for patients with raised stromal MMP-9 was 47.5 months (95% CI 41.8 – 53.2) compared with 54.1 months (95% CI 49.9 – 58.4) in the low stromal MMP-9 group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	47	45	44	41	22	20	6
Low	48	46	41	40	9	7	6

Figure 4.53: The relationship between stromal MMP-9 expression and overall survival in patients with colorectal cancer (p=0.064)

Kaplan-Meier curve demonstrating the proportion of patients surviving stratified by stromal MMP-9 *expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.*

On Cox univariable regression analysis stromal MMP-9 was not significantly associated with poor overall survival when dichotomised as a categorical variable (HR 2.35 (95% CI 0.93 – 5.96), p=0.072) however, the hazard ratio was significant when stromal MMP-9 was included as a continuous variable (HR 1.02 (95% CI 1.00 – 1.03), p=0.050). In addition to the associations with survival, stromal MMP-9 did not predict death during the follow up period as a categorical variable (AUC of 0.59 (95% CI 0.45 – 0.73, p=0.209), ROC analysis) or continuous variable (AUC of 0.61 (95% CI 0.49 – 0.74, p=0.119), ROC analysis), (figure 4.54).

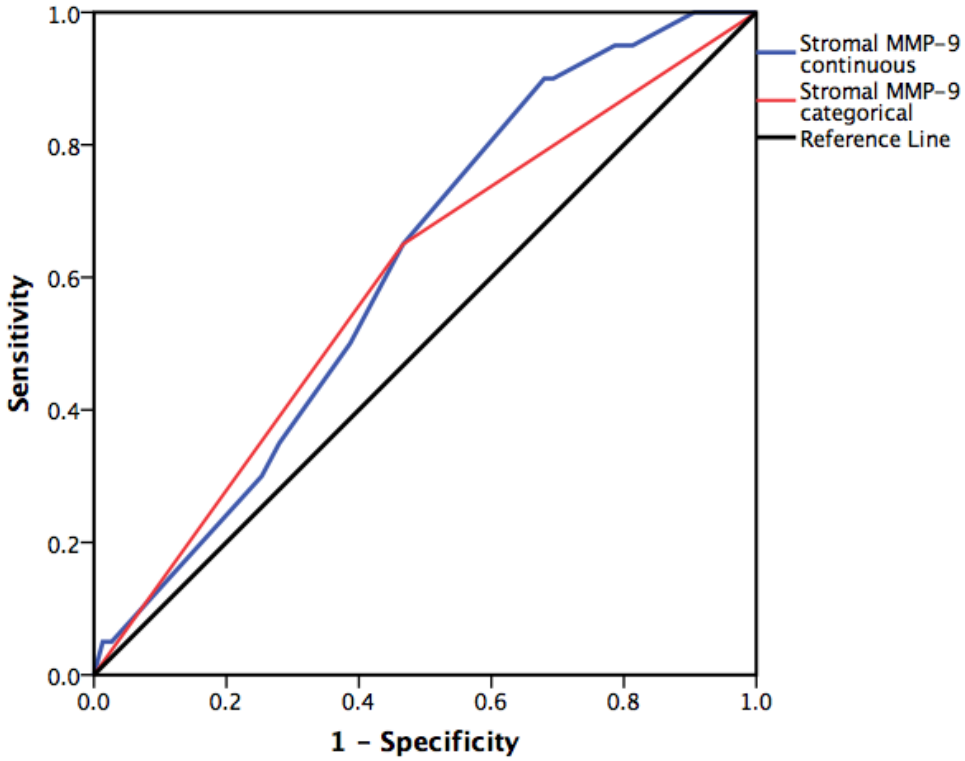


Figure 4.54: Predictive value of stromal MMP-9 in identifying patients who will die during follow-up

(A) Receiver-operator-characteristic curve demonstrating the predictive value of stromal MMP-9 *in identifying patients who will die during follow-up.*

4.4.9.6 Univariable overall survival - serum MMP-9 expression

Serum MMP-9 measurements were not higher in patients who died during follow-up (p=0.108, Mann-Whitney U, figure 4.55). The median for patients who died was 126.34 ng/ml (IQR 0.00 – 282.62 ng/ml) compared with 58.46 ng/ml (IQR 0.00– 154.32 ng/ml) in the alive group.

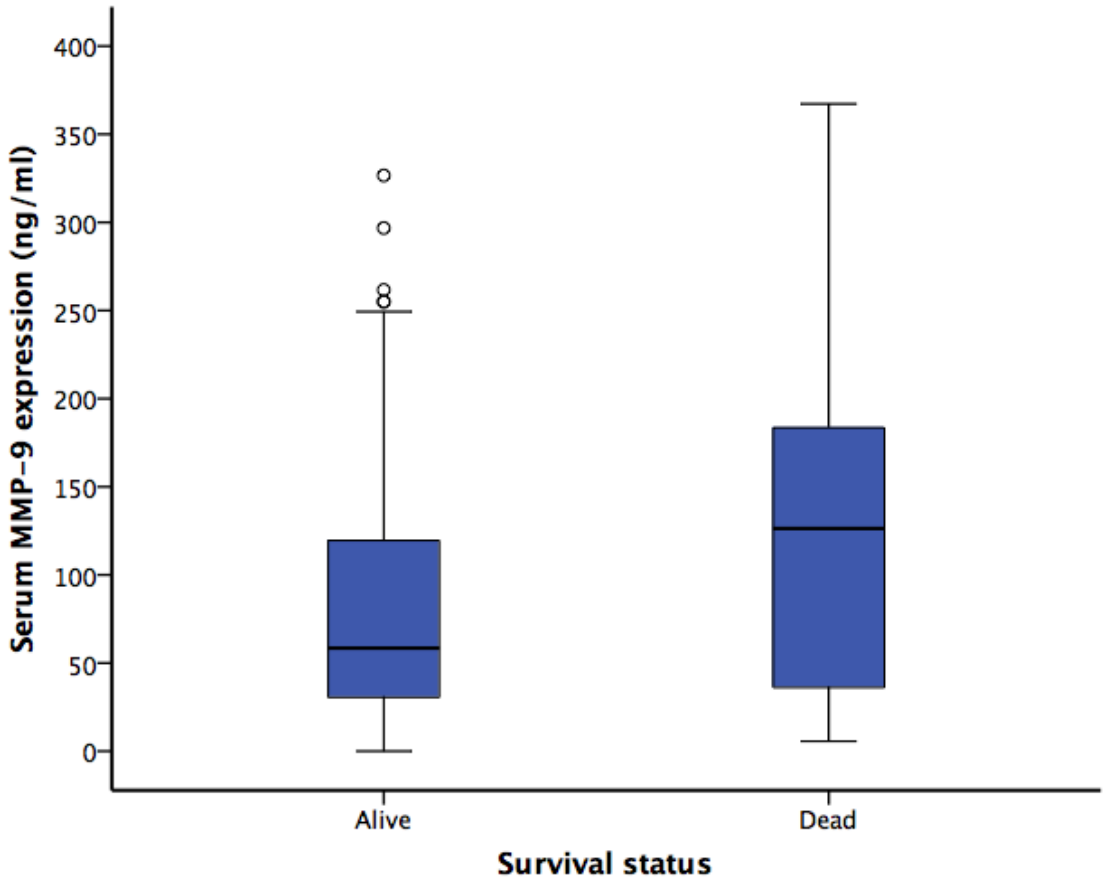
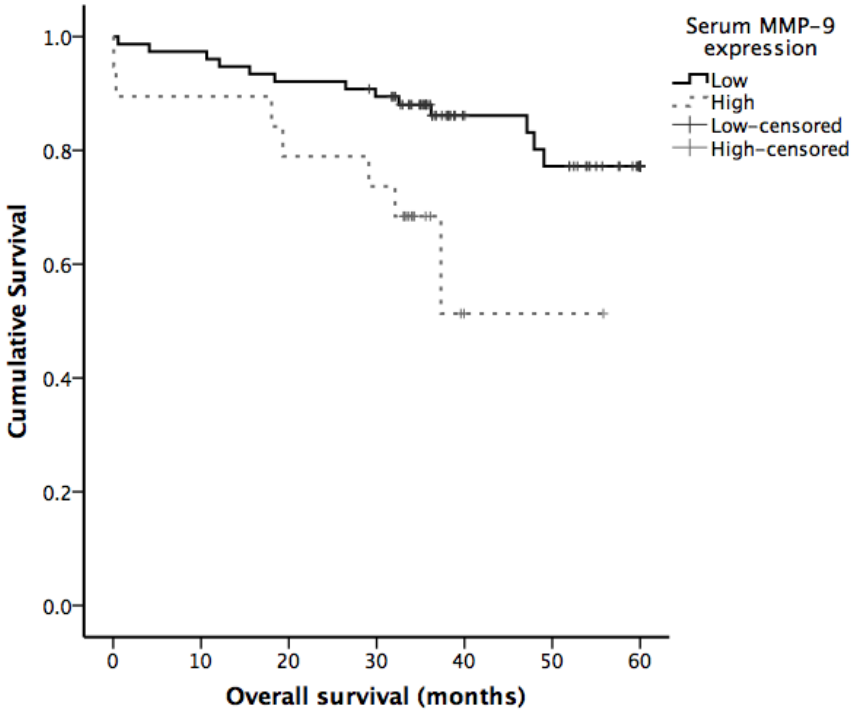


Figure 4.55: The distribution of serum MMP-9 measurements in patients stratified by survival status (p=0.108)

On logrank analysis, raised serum MMP-9 expression was not associated with poor overall survival (p=0.012, figure 4.56). The mean survival for patients with a raised serum MMP-9 was 40.2 months (95% CI 30.7 – 49.8) compared with 53.3 months (95% CI 49.9 – 56.8) in the low serum MMP-9 group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	76	74	70	67	31	26	12
High	19	17	15	14	1	1	0

Figure 4.56: The relationship between serum MMP-9 expression and overall survival in patients with colorectal cancer (p=0.012)

Kaplan-Meier curve demonstrating the proportion of patients surviving stratified by serum MMP-9 *expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.*

On Cox univariable regression analysis raised serum MMP-9 expression was associated with poor overall survival when dichotomised as a categorical variable (HR 3.16 (95% CI 1.22 – 8.15), p=0.018) and as a continuous variable (HR 1.01 95% CI 1.00 – 1.01, p=0.008). In addition to the associations with survival, serum MMP-9 did not predict death during follow-up as a categorical variable (AUC of 0.60 (95% CI 0.45 – 0.74, p=0.193), ROC analysis) or continuous variable (AUC of 0.62 (95% CI 0.47 – 0.77, p=0.108), ROC analysis), (figure 4.57).

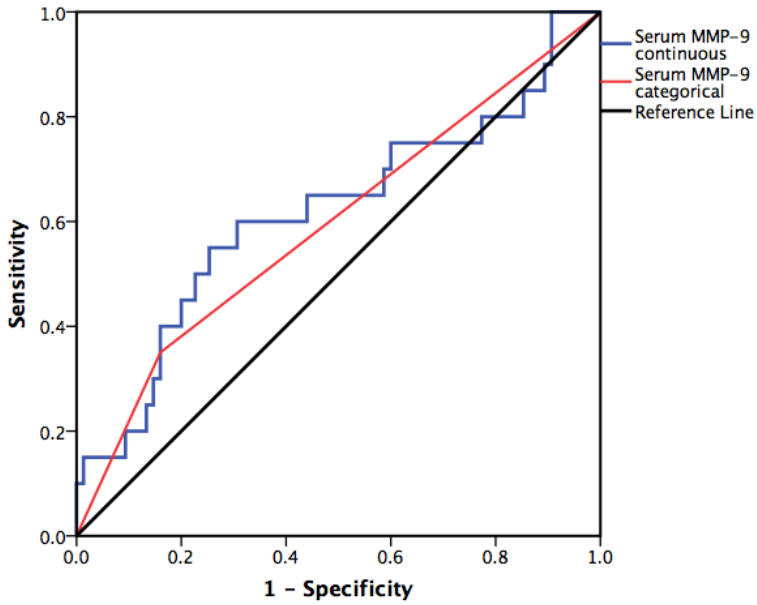


Figure 4.57: Predictive value of serum MMP-9 in identifying patients who will die during follow-up

Receiver-operator-characteristic curve demonstrating the predictive value of serum MMP-9 in identifying patients who will die during follow-up

4.4.9.7 Multivariable recurrence-free survival

Proportionality assumptions testing

All the studied variables underwent Cox proportional hazards assumption testing as described previously.

Univariable survival analysis

On univariable analysis, only N-stage (when compared with N0, N1 HR 3.52 (95% CI 1.07 – 11.54) and N2 (HR 5.96 (95% CI 1.60 – 22.24), p=0.020) and raised serum MMP-9 (HR 1.59 (95% CI 0.57 – 4.47), p=0.021) was associated with poor recurrence-free survival (table 4.9).

Multivariable analysis

It has been suggested that at least 10 - 25 events are required for each variable in a multivariable model. Based on the number of events noted above, no meaningful results can be generated by undertaking multivariable survival on this cohort of 95 patients. The relatively low number of patients and associated recurrence events (n=15) makes the chance of incurring a type 1 or type 2 reporting error highly likely. Therefore, multivariable survival analysis has not been performed on this cohort of patients.

Table 4.9: The relationships between MMP-9 expression, clinicopathological factors and recurrence-free survival: univariable analysis

	Univariable analysis		
	Coefficient	p-value	Hazard ratio (95% CI)
Age	-0.002	0.929	1.00 (0.95 - 1.05)
Sex			
Female			1
Male	0.806	0.168	2.24 (0.71 – 7.03)
Tumour site			
Colon			1
Rectum	-0.229	0.722	0.80 (0.22 – 2.82)
Tumour site			
Right	(0.000)	0.908	1
Left	-0.155		0.86 (0.26 – 2.85)
Rectum	-0.284		0.75 (0.20 – 2.84)
T stage			
1	(0.000)	0.473	1
2	9.212		10017.65 (0.00 – 3.45e137)
3	10.196		26790.72 (0.00 – 9.18e137)
4	10.799		48965.89 (0.00 – 1.68e138)
N stage			
0	(0.000)	0.020	1
1	1.258		3.52 (1.07 – 11.54)
2	1.785		5.96 (1.60 – 22.24)
TNM stage			
I	(0.000)	0.133	1
II	9.978		21539.76 (0.00 – 6.14e93)
III	11.078		64761.62 (0.00 – 1.84e94)
Differentiation			
Well/Moderate			1
Poor	1.300	0.087	3.67 (0.83 – 16.27)
Serum CRP			
Normal			1
High	-0.294	0.648	0.75 (0.21 – 2.64)
Serum albumin			
Normal			1
Low	-0.280	0.631	0.76 (0.24 – 2.37)
Cytoplasmic MMP-9			
Low			1
High	0.628	0.233	1.87 (0.67 – 5.27)
Stromal MMP-9			
Low			1
High	0.464	0.379	1.59 (0.57 – 4.47)
Serum MMP-9			
Low			1
High	1.215	0.021	3.37 (1.20 – 9.48)

4.4.9.8 Multivariable overall survival

Proportionality assumptions testing

All the studied variables underwent Cox proportional hazards assumption testing as described previously.

Univariable survival analysis

On univariable analysis, only serum MMP-9 (HR 3.16 (95% CI 1.22 – 8.15), p=0.018) was associated with poor overall survival. Stromal MMP-9 (HR 2.35 (95% CI 0.93 – 5.96), p=0.072) showed a trend towards an association with overall survival (p<0.1), however this was not statistically significant (table 4.10).

Multivariable analysis

It has been suggested that at least 10 - 25 events are required for each variable in a multivariable model. Based on the number of events noted above, no meaningful results can be generated by undertaking multivariable survival on this cohort of 95 patients. The relatively low number of patients and associated death events (n=20) makes the chance of incurring a type 1 or type 2 reporting error highly likely. Therefore, multivariable survival analysis was not been performed on this cohort of patients.

Table 4.10: The relationship between MMP-9 expression, clinicopathological factors and overall survival: univariable and multivariable analysis

	Univariable analysis		
	Coefficient	p-value	Hazard ratio (95% CI)
Age	0.015	0.503	1.02 (0.97 - 1.06)
Sex			
Female			1
Male	-0.163	0.722	1.18 (0.48 – 2.88)
Tumour site			
Colon			1
Rectum	0.244	0.618	1.28 (0.49 – 3.33)
Tumour site			
Right	(0.000)	0.808	1
Left	-0.381		0.68 (0.22 – 2.15)
Rectum	-0.121		0.89 (0.31 – 2.55)
T stage			
1	(0.000)	0.379	1
2	-0.539		0.58 (0.10 – 3.50)
3	-0.782		0.46 (0.10 – 2.13)
4	0.088		1.09 (0.22 – 5.42)
N stage			
0	(0.000)	0.147	1
1	-0.519		0.60 (0.17 – 2.09)
2	0.910		2.48 (0.80 – 7.68)
TNM stage			
I	(0.000)	0.954	1
II	-0.173		0.84 (0.26 – 2.73)
III	-0.072		0.93 (0.72 – 3.18)
Differentiation			
Well/Moderate			1
Poor	0.553	0.592	1.74 (0.23 – 13.19)
Serum CRP			
Normal			1
High	0.748	0.102	2.11 (0.86 – 5.17)
Serum albumin			
Normal			1
Low	-0.125	0.798	0.88 (0.34 – 2.30)
Cytoplasmic MMP-9			
Low			1
High	0.569	0.215	1.77 (0.72 – 4.34)
Stromal MMP-9			
Low			1
High	0.854	0.072	2.35 (0.93 – 5.96)
Serum MMP-9			
Low			1
High	1.149	0.018	3.16 (1.22 – 8.15)

4.5 Discussion

4.5.1 Summary of the novel results

The main novel finding within this chapter is the association of serum MMP-9 expression with poor survival. Furthermore, serum MMP-9 was associated with stromal and cancer cell MMP-9 expression.

4.5.2 Strengths and limitations

4.5.2.1 Strengths

This study has several strengths including the sample size of the validation cohort, the follow up data and treatment standards, which have been discussed previously. In addition to these, blood markers for CRP, albumin and MSI analysis were performed in an accredited NHS laboratory.

4.5.2.2 Limitations

Data sampling, power and survival modeling

The limitations regarding statistical power during the sub-division of the validation cohort, the relative weakness of the survival endpoints and the absence of previously reported prognostic clinicopathological factors in the multivariable model have been discussed previously. In addition, the sample size of cohort 3 is also associated with a significant risk of type II errors. This may explain why factors that are normally associated with poor outcome did not reach statistical significance.

Tissue preparation and scientific techniques

The difficulties and limitations relating to tissue dissection, tumour sampling and the accurate evaluation of biomarker expression have been discussed previously. The immunohistochemistry technique is associated with inherent limitations. These have been discussed previously, however, they relating to non-specific binding of the primary antibody and secondary amplification molecules to non-targeted proteins and the nature of immunohistochemistry quantifications is open to human error. Immunohistochemistry is fundamentally very difficult to quantitate and the most reliable method would be to undertake grain counting using a radioactive label, which would only quantify the antibody and not the antigen. A further specific immunohistochemistry limitation of the studies

presented in this chapter is that the MMP-9 antibody used cannot differentiate between the active and inactive forms of the protein. Unfortunately, given the structure of the MMP-9 protein there are no antibodies available, which can identify the active form of the protein and therefore even low expressing tumours, may contain a higher proportion of active MMP-9. A possible solution would be to use western blot to identify the presence of active (88 kDa) MMP-9 in laser captured cancer cell lysates, however, this method of protein identification is not reliably accurate when trying to determine expression levels and a robust and validated method is needed.

4.5.3 Discussion of the results

4.5.3.1 MMP-9 expression and survival

This is the first study to show that serum MMP-9 expression is associated with poor recurrence free survival and therefore there are no confirmatory studies available to validate this finding. Previous studies examining prognosis in patients with colorectal cancer have concentrated on tumoural expression of MMP-9. Cytoplasmic MMP-9 expression was not associated with survival in the validation cohort and the associations observed in the training cohort may represent a type I error. A metanalysis of 9 studies containing a total of 1674 patients showed that tumour MMP-9 overexpression was associated with poorer recurrence-free and overall survival (Li, 2013). Although Chun *et al* observed these associations with survival, not all studies were significant. Furthermore, the metanalysis was limited by including studies that used either immunohistochemistry or PCR methods for quantifying MMP-9 expression. In addition, the antibodies utilised varied and included those that are commercially available and those privately manufactured as part of a research collaboration. Given the heterogeneity in immunohistochemistry methodology and immunoreactivity quantification the reported observations between tumour MMP-9 expression and survival, in the medical literature, must be interpreted with caution and similar problems may explain, in part, why so few biomarkers become routinely used in clinical practice.

The failure to observe a significant relationship between MMP-9 expression in the cytoplasm and survival in the validation cohort could be explained by the MMP-9 antibody binding to both active and inactive forms of the protein or the inability to score stroma expression given the use of a TMA for tumour tissue quantification. In the serum/tumour cohort, stromal immunoreactivity for MMP-9 was trending towards being significantly

associated with poor overall survival (HR 2.35 (95% CI (0.93 – 5.96); p=0.072) however this may represent a type II error given the relatively small sample number.

4.5.3.2 MMP-9 expression and colorectal cancer

The results of this study suggest that MMP-9 plays an important role in tumour progression with serum MMP-9 being associated with advanced TNM stage. The findings by Zeng *et al* support these observations when they found that MMP-9 was higher in metastasis compared to the primary (Zeng, 1995) and higher levels were also expressed in more advanced tumours (Zeng, 1995). These studies used mRNA levels for MMP-9 quantification, which incorporates all tumoural areas and thus all sources of MMP-9 production. Our method of quantification specifically looked at tumour cell cytoplasm in the validation cohort and tumour cell cytoplasm and stroma in the serum/tissue cohort. It is possible that MMP-9 exerts its biological effect in the stroma as degradation of the connective tissue will help with tumour cell invasion and metastasis and this may be the optimum tumoral region for biomarker quantification; this requires further study.

Serum MMP-9 expression in colorectal cancer has been studied previously, however, this was the first study to examine its prognostic value (Tutton, 2003). Despite exhibiting powerful prognostic value, we found no significant difference between serum MMP-9 in patients and controls. Although this seems paradoxical, similarities can be drawn to other biomarkers that are not cancer specific such as the neutrophil-lymphocyte ratio, CRP and hypoalbuminaemia. It is therefore possible that MMP-9 expression is not cancer specific and may be highly expressed because of other physiological processes as seen with other serum biomarkers such as CRP. This may explain why Wilson *et al* observed that MMP-9 had no value as a screening tool for patients who were FOB positive on bowel screening (Wilson, 2006) yet there was an associations with poor survival in this study.

We observed that MMP-9 was expressed in the cancer cells, stroma and the serum of patients. Physiologically MMP-9 is secreted by immune cells to aid tissue remodelling and MMP-9 has been observed in macrophages at the tumour margin in primary and metastatic disease (Zeng, 1995; Zeng, 1998). Expression of MMP-9 within the cancer cells has been observed previously and our results support these findings (Yang, 2014), however, it remains unclear what biological effect cytoplasmic MMP-9 has on the tumour cell. It is possible that MMP-9 is secreted by the cancer cell to degrade the connective tissue closest to the tumour, however, the hypothesis that cancer cells make their own

MMP-9 is controversial. It has been suggested that MMP-9 in the tumour cell originated from fibroblasts and immune cells in the stroma. It was thought that MMP-9 in the cancer cell was taken up from the microenvironment and therefore purely reflected stromal degradation activity (Roeb, 2001; Collins, 2001). Interestingly, recent advances in tissue microdissection with laser capture have revealed that cancer cells express mRNA for MMP-9 (Lubbe, 2006). This suggests that the tumour cell, stroma and circulation may all be sites of MMP-9 production and identifying which expression site exerts the maximal biological effect will help translate MMP-9 into clinical practice.

4.5.3.3 The influence of inflammation on MMP-9 expression

As described above, MMP-9 found in the stroma has been thought to be secreted by immune cells to aid tissue remodeling. This makes sense given that the immune cells would have to degrade the connective tissue to move through the stoma towards the invasive margin of the cancer. Despite this, there was no significant association between the Klintrup score and tumour cell cytoplasmic MMP-9 expression. This supports the notion that stromal and cancer cell MMP-9 expression is not dependent on the same regulatory factors, however, MMP-9 expression in the circulation, cytoplasm and stroma were all closely related as evidence by the significant correlations observed in the chapter.

In other disease processes, MMP-9 has been implicated in tissue remodeling as part of the inflammatory response. It is plausible that there is an association between the SIR and MMP-9 expression in the cancer cell. The mechanisms leading to cancer MMP-9 production remain unknown, however, Il-6 through COX2 and JAK/STAT activation has been implicated in regulating MMP-9 production in macrophages (Kothari, 2013). In human colorectal cancers, the evidence is not as compelling, however, Liu *et al* observed that Il-6/STAT3 mediated induction of fos-related antigen 1 resulted in higher expression of MMP-9 in colorectal cancer cell lines (Liu, 2015). This observation is unlikely to represent true causality, as in vivo; there will be multiple stimuli and inhibitors of the MMP-9 pathway. Despite this, CRP was associated with cytoplasmic MMP-9 ($p=0.028$) in the validation cohort and the relationship between the SIR and MMP-9 expression requires further study.

4.5.3.4 MMP-9 as a prognostic and predictive biomarker

Cytoplasmic MMP-9 in the tumour cell was not associated with survival in the validation cohort. The potential reasons for this have been discussed above, however, this observation

has a significant impact on how MMP-9 can be translated into a useful prognostic or predictive biomarker. Studies that observed a significant relationship between MMP-9 expression and survival simply quantified MMP-9 mRNA levels in the homogenate. This method of biomarker quantification is rather crude as the homogenate will include both cancer and non-cancer tissue. This poses difficulties for the development and validation process as there are no easy and reliable methods for controlling the proportions of non-cancer and cancer tissue in samples from different patients. Ultimately, the origin of the MMP-9 within the stroma is an important factor in validating MMP-9 as a potential biomarker. Allowing for the possibility that MMP-9 also plays an important role in signal transduction within the cancer cell, the stroma is the most likely site of MMP-9 biological activity. Unfortunately the results presented in this chapter cannot shed light on the sources of stroma MMP-9 and this requires further study.

Looking at current practice, the majority of biomarkers are histological in nature and utilise IHC or FISH with specific localisation to subcellular areas of the tumour. Since the MMP-9 antibody binds to both active and inactive forms of the protein, a process for identifying tumours with high expression of active MMP-9 is needed. A possible method could be to combine IHC and western blot analysis as the active form of MMP-9 has a lower molecular weight of 88 kDa, however this approach has never been tested. Despite the rather non-quantitative character of IHC it is interesting to reflect that the assessment of oestrogen receptor status became substantially more reliable when the biochemical analysis of pieces of possible tumour was replaced by IHC when good antibodies became available which worked in paraffin sections. Therefore, the main limiting factor preventing the incorporation of MMP-9 into the staging process is the absence of a reliable method of quantification. Options for MMP-9 include IHC on full tissue sections with particular attention to the stroma or using ELISA on the patients' serum. The latter requires validation of the association between serum and tumour expression, however, it controls for proportional representation of MMP-9 in the serum; as one would anticipate venous blood samples to have a near uniform concentration of the protein across the whole blood volume. Given the associations observed in this chapter between serum MMP-9 expression and survival, this may be a useful solution to identifying which of the cancer related tissue components (tumour or venous blood) offers the most useful prognostic biomarker medium. Further studies tailored to specifically answer this question are needed before assessing the predictive value of MMP-9 as a cancer biomarker.

Despite the growing evidence suggesting that MMPs play an important role in cancer progression and metastasis, clinical trials of MMP inhibitors (MPI) have been

disappointing (Coussens, 2002a). To date there are no clinical trials looking at MMP inhibitors in colorectal cancer, however, there have been phase III trials in other cancers such as gastric, pancreatic and lung. Despite compelling preclinical data implicating MMP-9 in cancer progression, studies suggested no role for MPI in cancer treatment. Similar to HER2 positivity and Herceptin, it is possible that high MMP-9 expression in the serum or tissue may predict response to MPIs and retrospective sub-analysis may offer evidence for their use as novel adjuvant therapies.

4.5.4 Future direction

1. Serum MMP-9 in a larger cohort with tissue analysis using full tissue sections for cancer and stroma expression quantification
2. Through isolating the cancer cells and stroma by microdissection, performing western bloat analysis to identify tumours with high expressions of active MMP-9 and seeing if this helps strengthen the prognostic value of cytoplasmic and stromal MMP-9
3. Looking at the effect of MMP-9 inhibitors on tumour MMP-9 expression and what happens to tumour behaviour
4. Trial of MMP-9 inhibitors in patients with high expression of MMP-9

4.5.5 Conclusion

MMP-9 in the serum may offer a useful prognostic biomarker with tumoural MMP-9 expression acting as a predictive biomarker for MMP inhibitors. Given that the IHC process uses an antibody for MMP-9 that binds to active and inactive forms of the MMP-9 protein, an additional method possibly including the identification of active MMP-9 through western blot analysis may be a possible solution. Studying the association of serum and tumoural MMP-9 expression, clinicopathological factors and survival in an adequately powered cohort will add further evidence for introducing MMP-9 into the prognostication process or not. Furthermore, serum or tumoural MMP-9 may help identify patients who will need and benefit from MMP inhibitors and this is worthy of further work.

5. Src family kinases and colorectal cancer

5.1 Introduction

The mechanisms by which colorectal cancer cells locally invade through the bowel and establish metastases remain uncertain. Tumour cell proliferation, loss of apoptosis and degradation of connective tissue are all important features of cancer cell behaviour. Loss of cell-cell adhesion and cell motility are also important and signalling pathways relating to adhesion and motility are also likely to play an important role in colorectal cancer progression and survival.

Src family kinases (SFKs) have been implicated in many adverse cancer cell behaviors including proliferation, apoptosis, invasion and cellular adhesion (Frame, 2002; Summy, 2003). SFKs comprise 9 family members BLK, C-SRC, FGR, FYN, HCK, LCK, LYN, YES, YRK. C-SRC has been the most investigated of all SFKs, but the role of other SFKs in cellular behaviors and their prognostic value remains largely unknown. The development of Src inhibitors, such as Dasatinib, has identified SFKs as a potential therapeutic target for patients at higher risk of a poor outcome. Unfortunately, clinical trials so far have not been promising but this may reflect inadequate patient selection.

The aims of this study were threefold. Firstly, to establish which of the Src family members were most highly expressed in colorectal cancers, we performed RT-qPCR on a historic cohort of frozen tumours and non-cancerous polyps. Secondly, to investigate associations between SFK expression and commonly recorded clinicopathological factors and survival, we assessed SFK expression in non-metastatic colorectal cancers employing a TMA. Finally, the effect Src inhibitor Dasatinib has on colorectal cancer cell proliferation, apoptosis and expression of SFKs were investigated.

5.2 Frozen tissue cohort

5.2.1 SFK expression in colorectal cancer

Twenty three (85%) patients with colon cancer were studied. With regards to cancer stage, 12 (44%) were Dukes B, 9 (33%) were Dukes C and 4 (15%) were Dukes D. Of all the specimens studied, 2 (7%) had high-grade dysplasia and were not considered cancers.

5.2.1.1 SFK mRNA expression in the whole cohort

Expression levels of SFK mRNA were quantified in all tissue samples (figure 5.1). BLK was the least expressed with LYN (159 fold) and SRC (83 fold) being the highest expressed SFKs when compared to the housekeeping gene. The aim of this study was to observe which family members were expressed and not to perform statistics to test for associations, due to the small number of patients available to study.

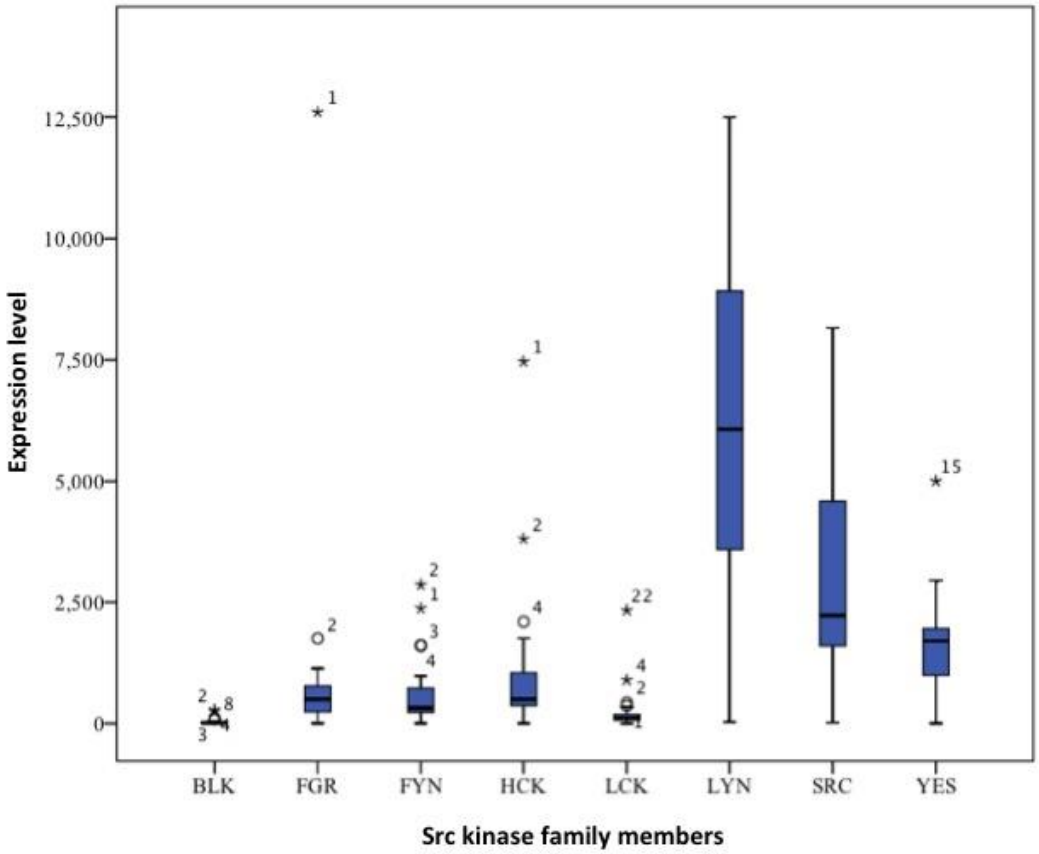


Figure 5.1: Distribution of SFK mRNA expression in all patients

Boxplot detailing the relative expression of all Src kinase family members in the entire cohort.

5.2.1.2 SFK mRNA expression stratified by Dukes' stage

Even although the cohort was not suitable for testing for statistical associations, we were able to look at trends in the data. FGR, HCK and FYN appeared to have an increase in expression from localised (stage II and III) to metastatic disease (stage IV) (Figure 6.2). Furthermore, LCK became less expressed from HGD to stage IV disease. The expression of LYN and Src did not appear to change across disease stages.

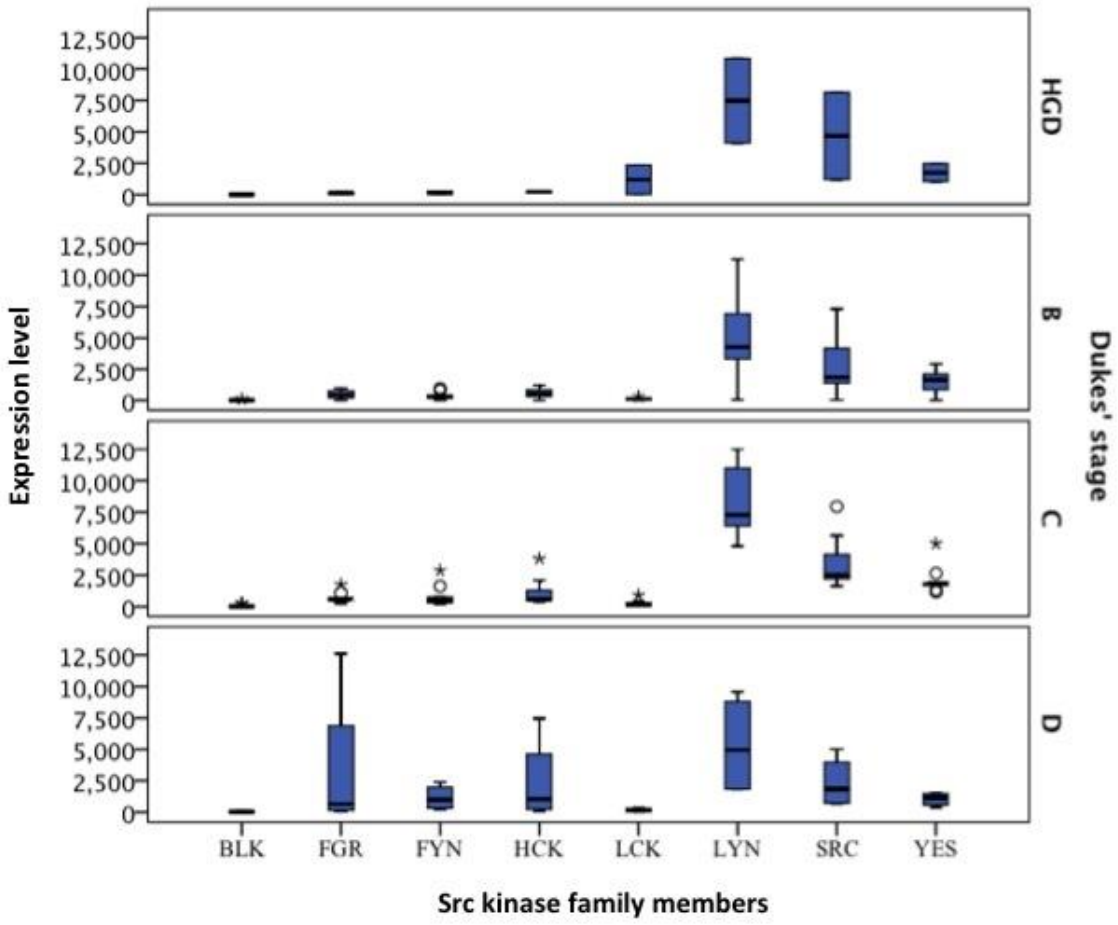


Figure 5.2: Distribution of SFKs mRNA expression across tumour stage

Boxplot detailing the relative expression of all SFK members across increasing tumour stage.

5.3 Training cohort (cohort 1)

5.3.1 Colorectal cancer FGR expression

Cancer cell FGR expression was evaluated using immunohistochemistry and scored using the weighted histoscore as previously described. Tumour cell membrane immunoreactivity was observed in 72.0% of patients, with 100.0% of tumours demonstrated cytoplasmic immunoreactivity and 92.3% of patients demonstrating nuclear immunoreactivity (figure 5.3).

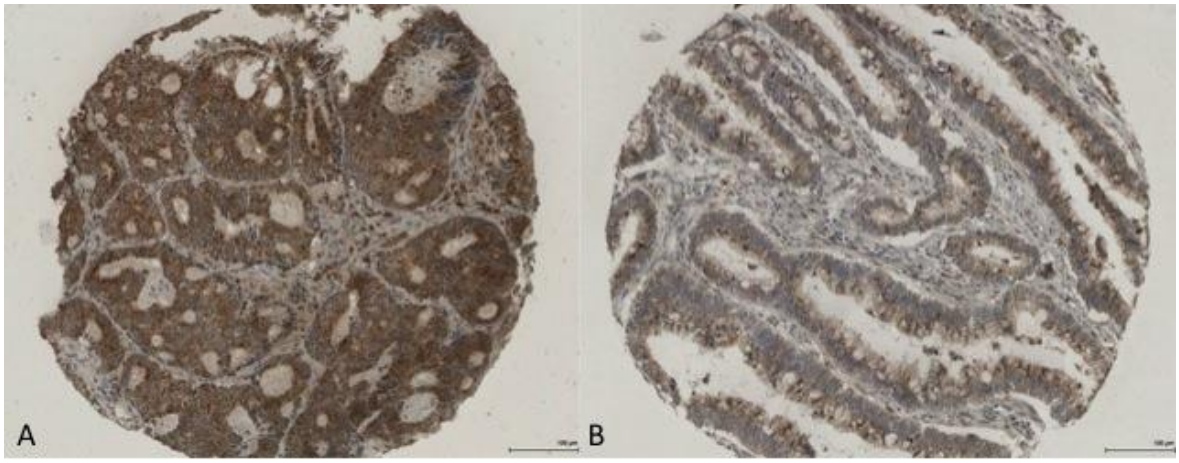
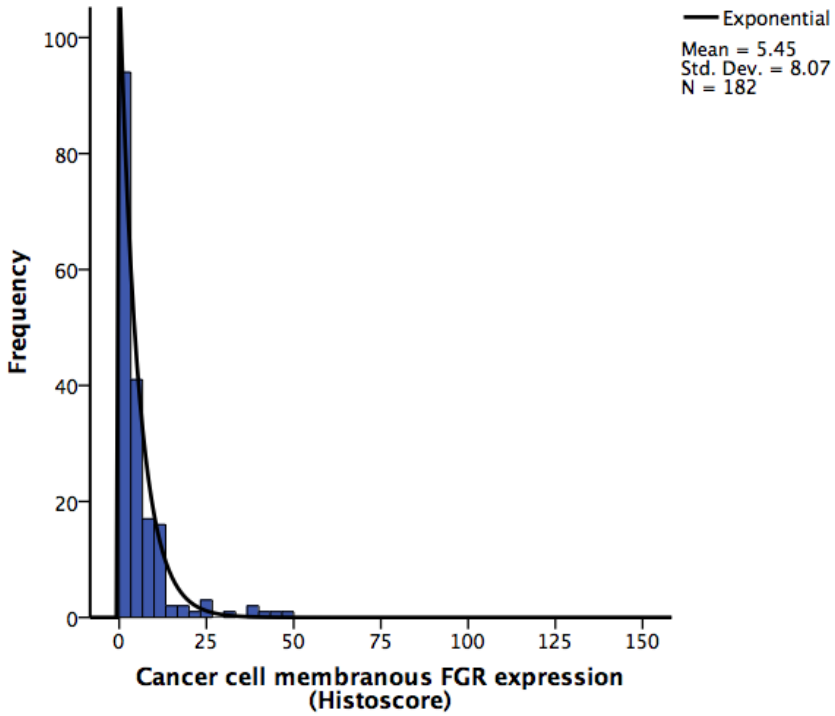


Figure 5.3: FGR Immunohistochemistry

Representative areas of cancer cells showing high and low FGR expression (A and B)

5.3.1.1 Cancer cell membranous FGR expression

Figure 3.1 demonstrates that the expression of tumour cell membranous FGR follows an exponential distribution (histogram, figure 5.4) with measurements ≥15.00 considered outliers (boxplot, figure 3.1b). The measurements ranged from 0.00 – 47.50 and had a median of 3.33 (IQR 0.00 - 10.21).



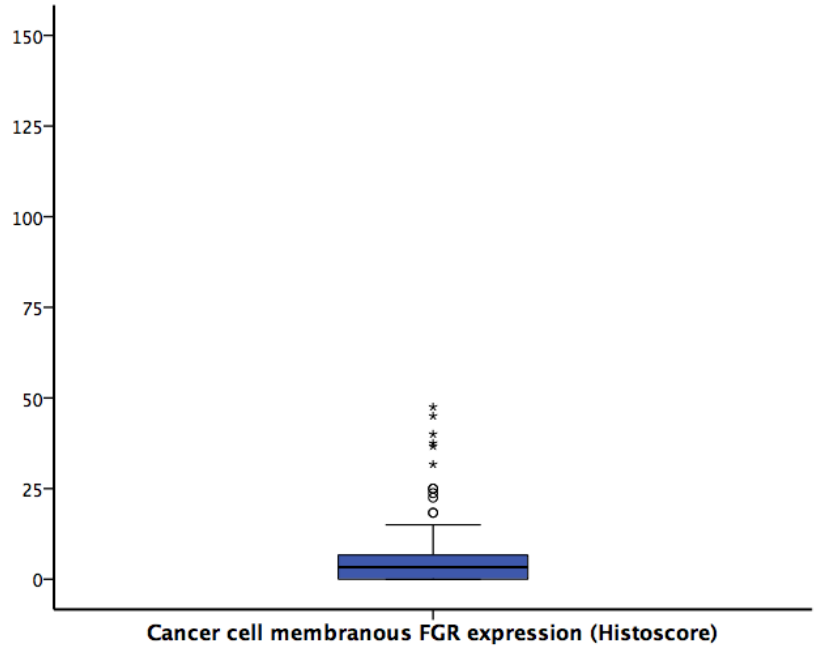
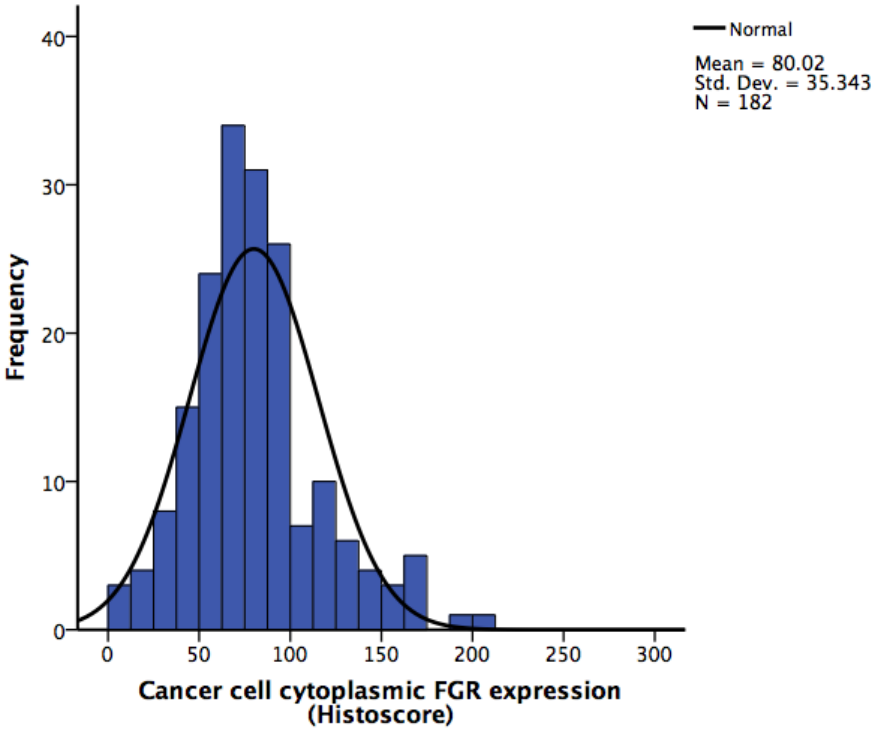


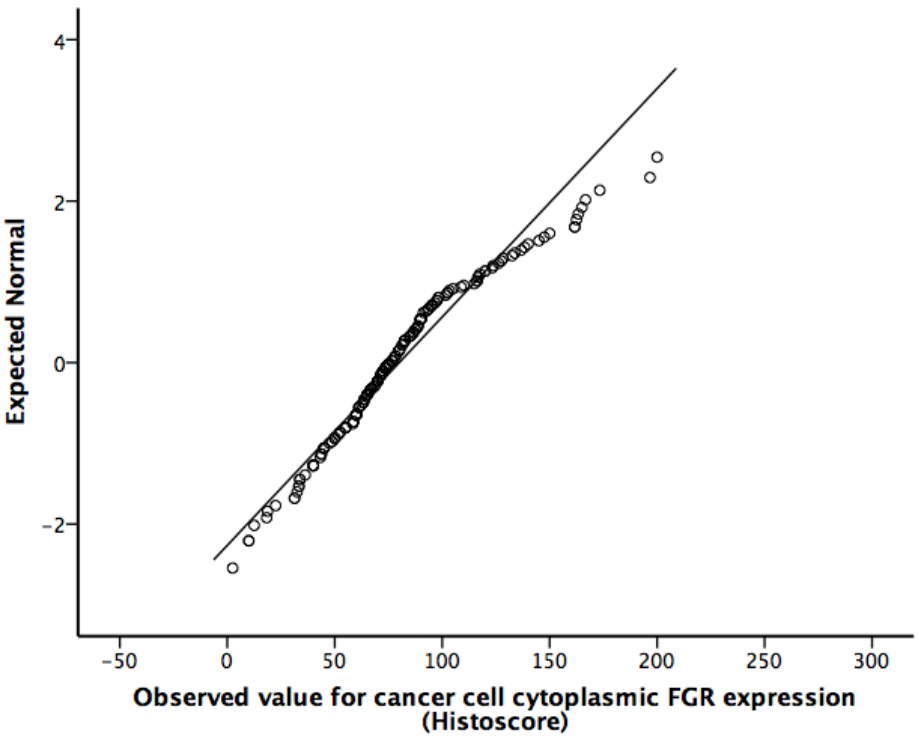
Figure 5.4: Distribution of measurements for cancer cell membrane FGR

Histogram demonstrating the distribution of cancer cell membrane FGR. Boxplot demonstrating the median measurement, interquartile range and outliers of tumour cell membrane FGR.

5.3.1.2 Cancer cell cytoplasmic FGR expression

Figure 5.5 demonstrates that the expression of tumour cell cytoplasm FGR does not precisely follow a normal distribution (histogram, figure 3.2), which is supported by a Shapiro-Wilk statistic of 0.955, df 182, $p<0.001$. The measurements ranged from 2.50 – 200.00 and had a median measurement of 75.84 (IQR 41.78 – 109.90).


Normal
Mean = 80.02
Std. Dev. = 35.343
N = 182
Frequency
40
30
20
10
0
0
50
100
150
200
250
300
Cancer cell cytoplasmic FGR expression
(Histoscore)


Expected Normal
4
2
0
-2
-50
0
50
100
150
200
250
300
Observed value for cancer cell cytoplasmic FGR expression
(Histoscore)

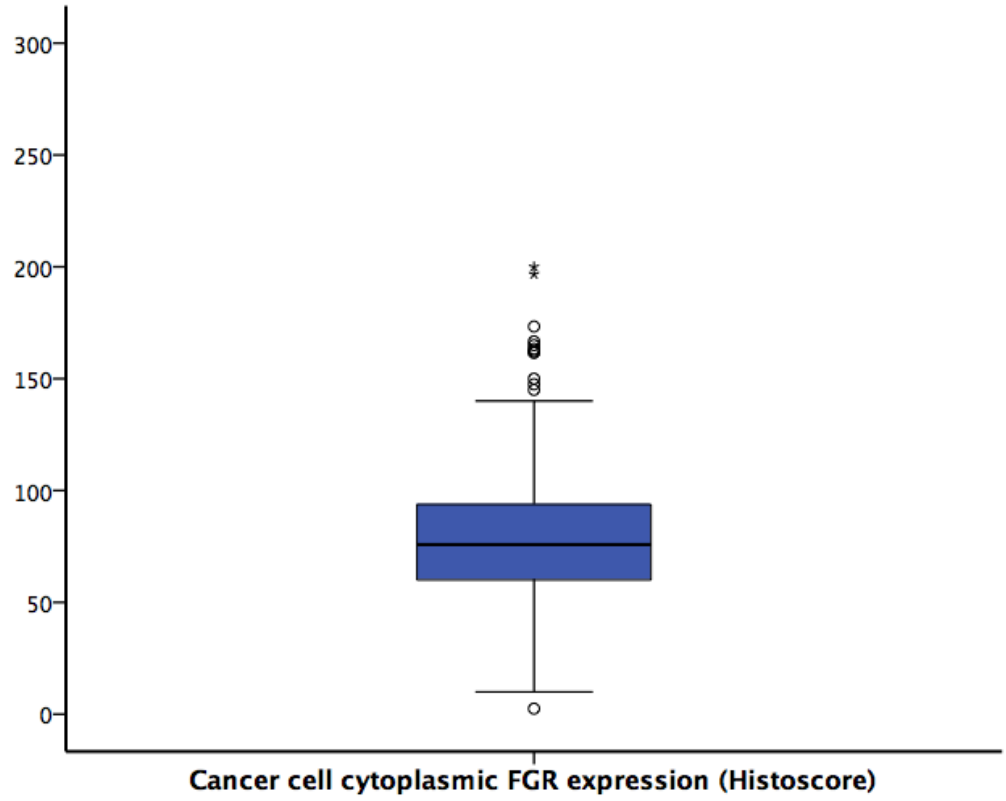


Figure 5.5*:* Distribution of measurements for cancer cell cytoplasmic FGR

Histogram demonstrating the distribution of cancer cell cytoplasmic FGR. Q-Q Plot of cancer cell cytoplasmic FGR measurements. Boxplot demonstrating the median measurement with interquartile range and outliers.

5.3.1.3 Cancer cell nuclear FGR expression

Figure 5.6 demonstrates that the expression of tumour cell membranous FGR follows an exponential distribution (histogram, figure 5.6) with measurements ≥35 considered outliers (boxplot, figure 5.6). The measurements ranged from 0.00 – 100.00 with a median of 10.00 (IQR 0.00 - 21.88).

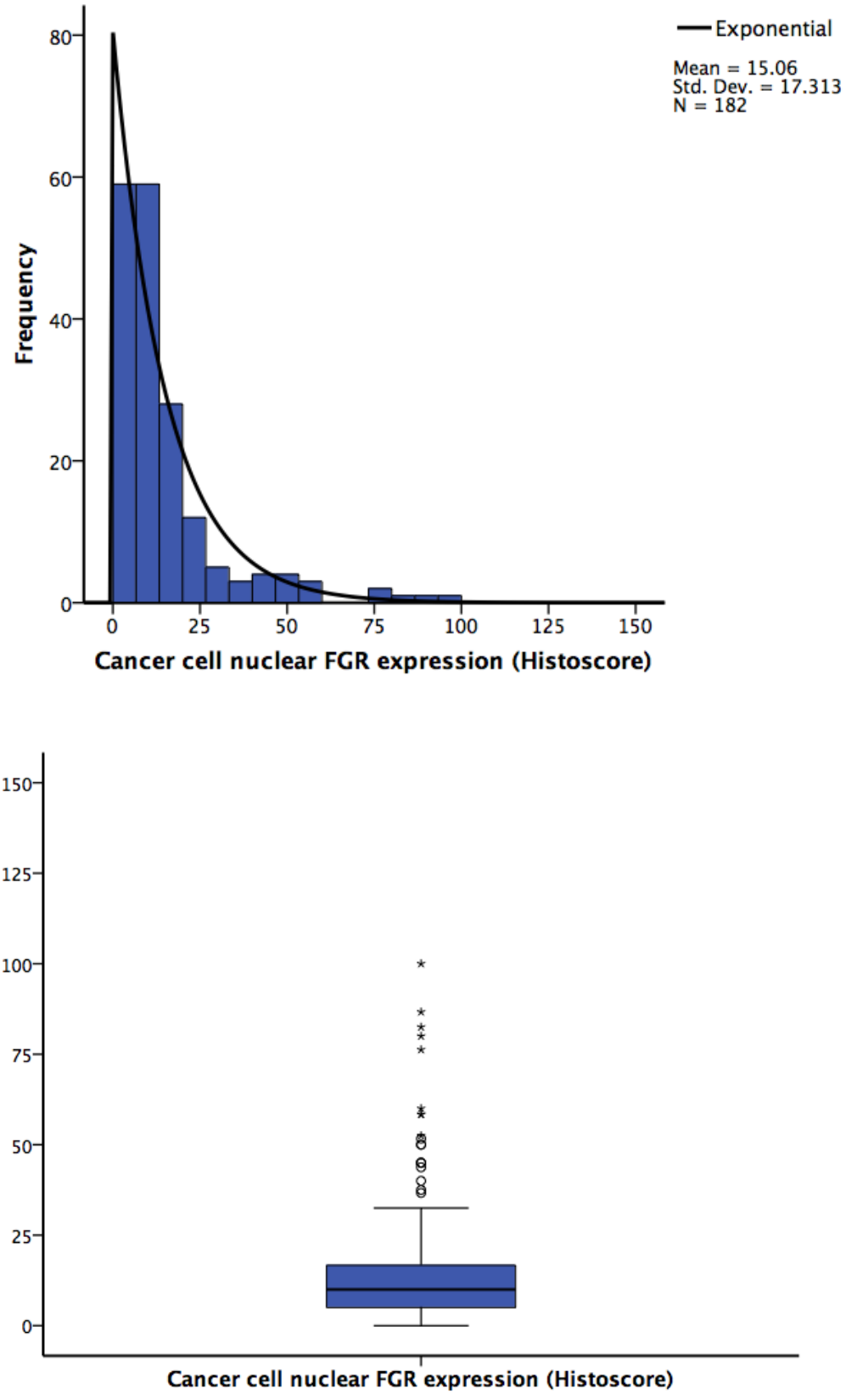


Figure 5.6: Distribution of measurements for cancer cell nuclear FGR
Histogram demonstrating the distribution of cancer cell nuclear FGR expression. Boxplot demonstrating the median measurement, interquartile range and outliers of cancer cell nuclear FGR.

5.3.2 Colorectal cancer HCK expression

Tumour cancer cell HCK expression was evaluated using immunohistochemistry and scored using the weighted histoscore as previously described. Membranous, cytioplasmic and nuclear expression of HCK was observed in all patients (figure 5.7).

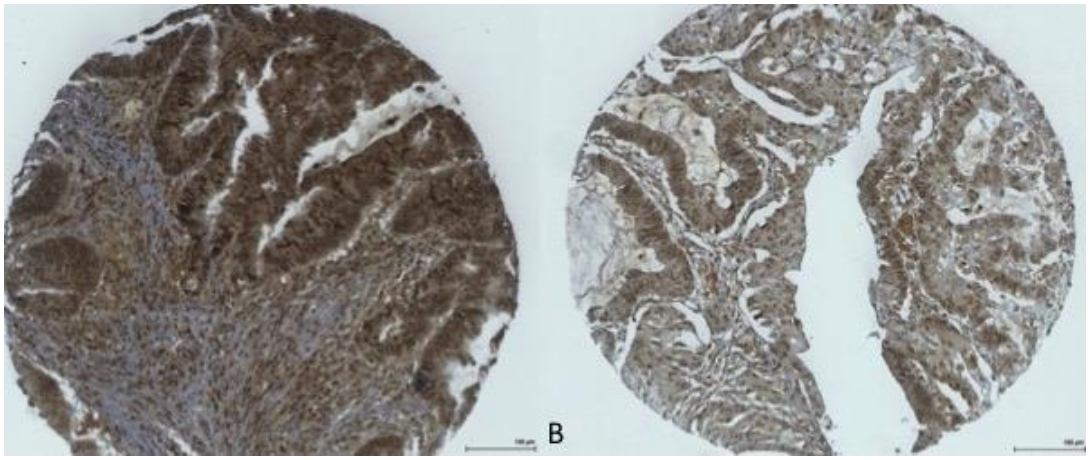
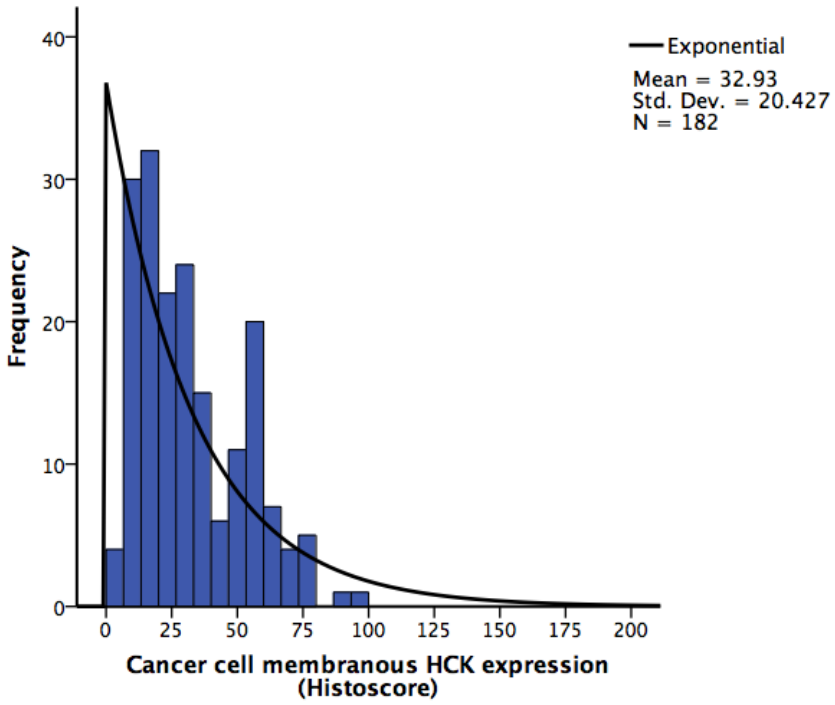


Figure 5.7: HCK Immunohistochemistry

Representative areas of cancer cells showing high and low HCK expression (A and B)

5.3.2.1 Cancer cell membranous HCK expression

Figure 5.8 demonstrates that the expression of tumour cell membranous HCK follows an exponential distribution (histogram, figure 5.8) with measurements ≥96.25 considered outliers (boxplot, figure 5.8). The measurements ranged from 5.00 – 96.25 and had a median of 28.75 (IQR 5.00 - 60.31).



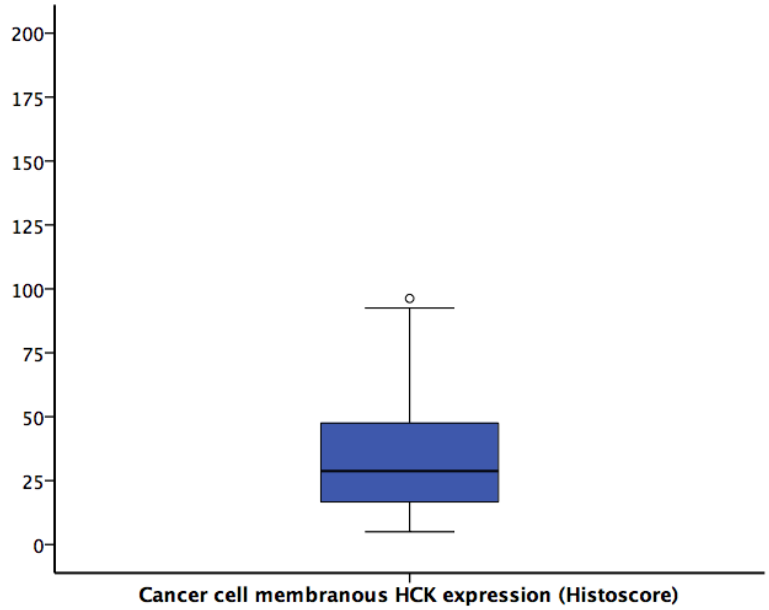
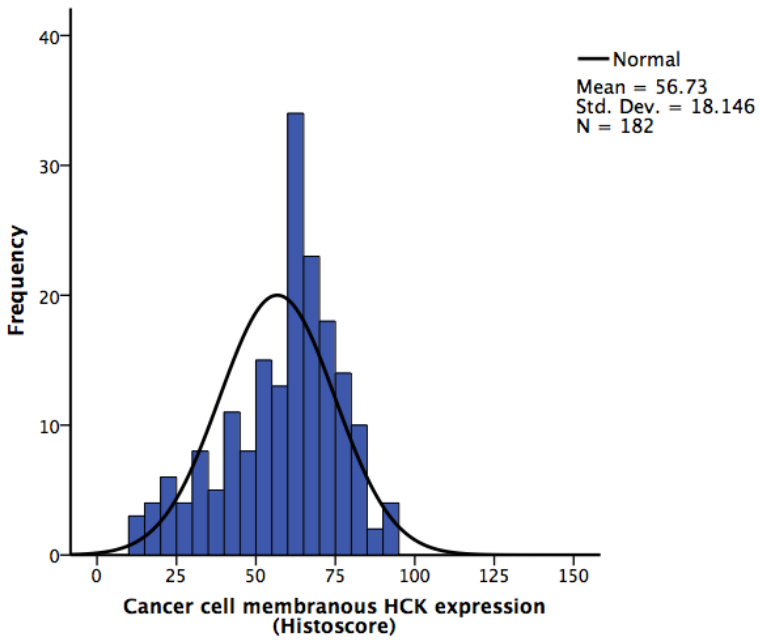


Figure 5.8: Distribution of measurements for cancer cell membrane HCK

Histogram demonstrating the distribution of cancer cell membrane HCK. Boxplot demonstrating the median measurement, interquartile range and outliers of cancer cell membrane HCK.

5.3.2.2 Cancer cell cytoplasmic HCK expression

Figure 5.9 demonstrates that cancer cell cytoplasm HCK expression does not precisely follow a normal distribution (histogram, figure 3.5), which is supported by a Shapiro-Wilk statistic of 0.953, df 182, p<0.001. The measurements ranged from 10.00 – 91.25 and had a median of 60.00 (IQR 36.66 – 83.33).



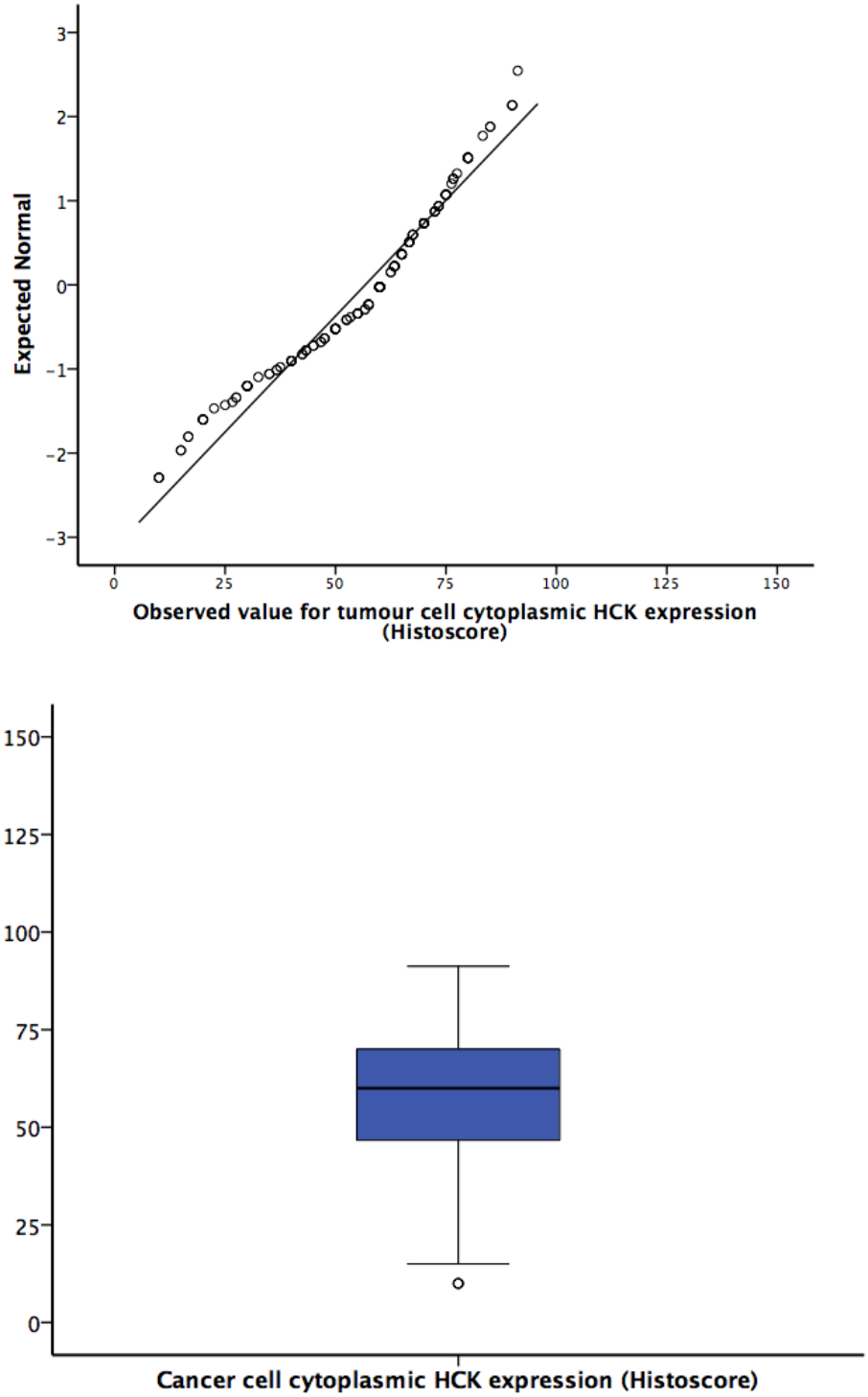


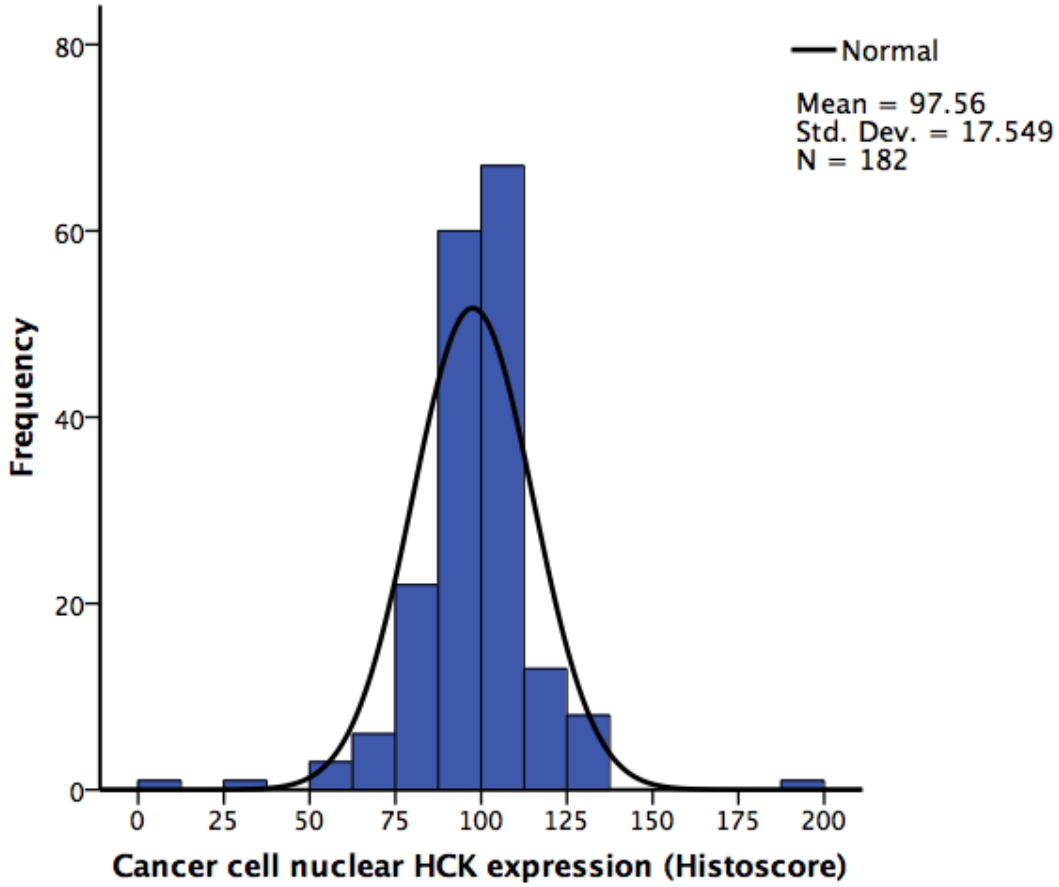
Figure 5.9: Distribution of measurements for cancer cell cytoplasmic HCK

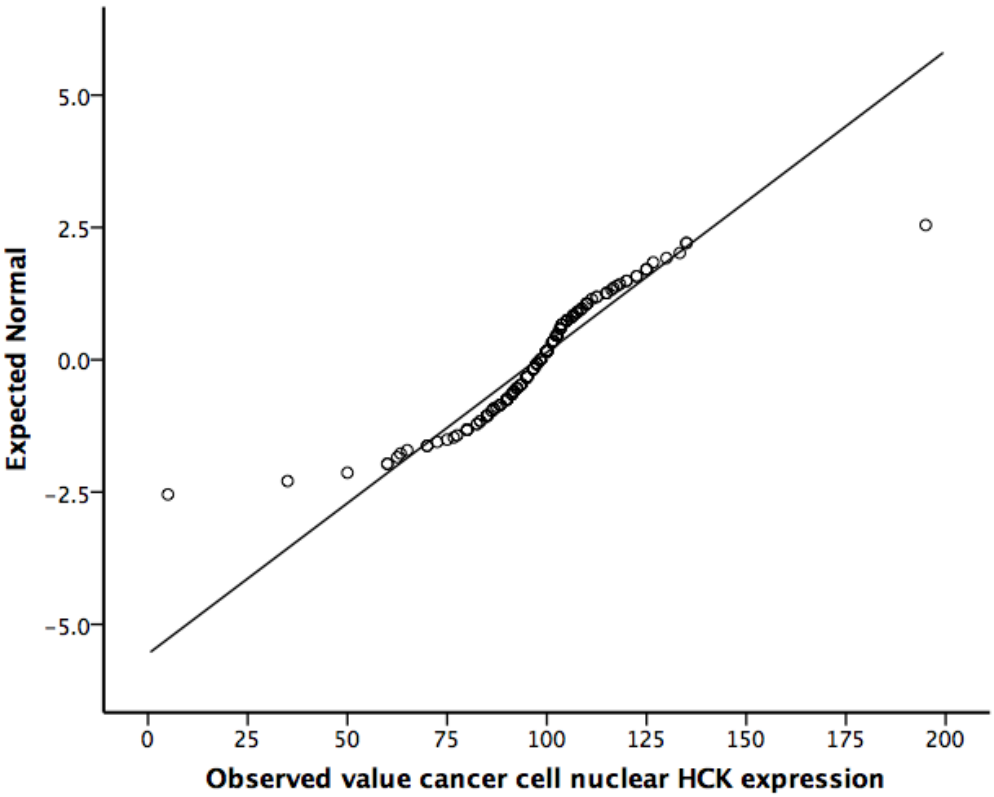
Histogram demonstrating the distribution of the cancer cell cytoplasmic HCK. Q-Q Plot of cancer cell cytoplasmic HCK measurements. Boxplot demonstrating the median measurement with interquartile range and outliers.

5.3.2.3 Cancer cell nuclear HCK expression

Figure 5.10 demonstrates that cancer cell nuclear HCK expression does not precisely follow a normal distribution (histogram, figure 5.10), which is supported by a Shapiro-

Wilk statistic of 0.868, df 182, p<0.001. The measurements ranged from 5.00 – 195.00 and had a median of 98.33 (IQR 85.52 – 111.14).





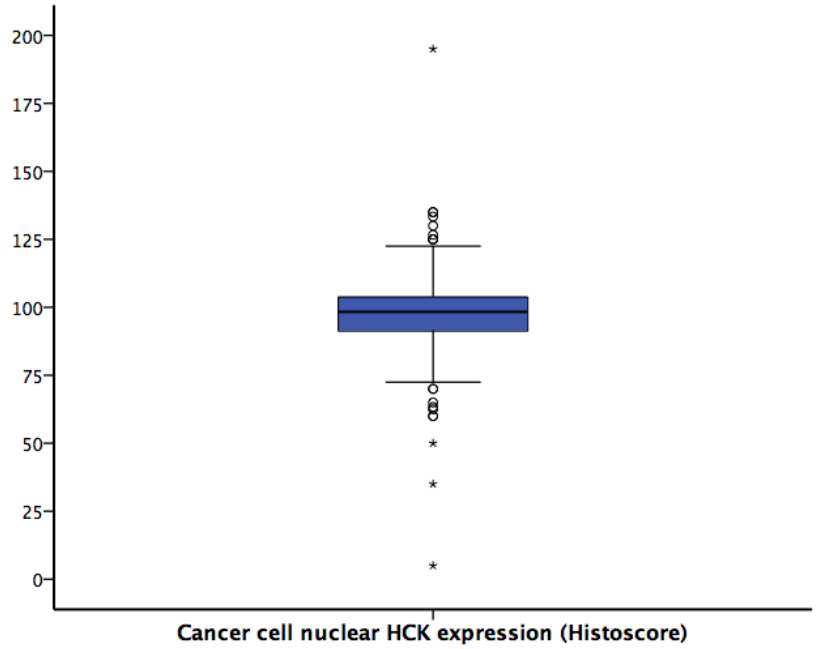


Figure 5.10: Distribution of measurements for cancer cell nuclear HCK

Histogram demonstrating the distribution of cancer cell nuclear HCK expression. Boxplot demonstrating the median measurement, interquartile range and outliers of cancer cell nuclear HCK.

5.3.3 Src activation – FAK (tyr 861) expression

The expression of FAK (tyr861) in the cancer cells within the tumour was evaluated using immunohistochemistry and scored using the weighted histoscore as previously described. Tumour cancer cell membrane immunoreactivity was not observed in any of the tumours, however, 15.9% of the tumours demonstrated cytoplasmic immunoreactivity and 99.5% of tumours demonstrating nuclear immunoreactivity (figure 5.11).

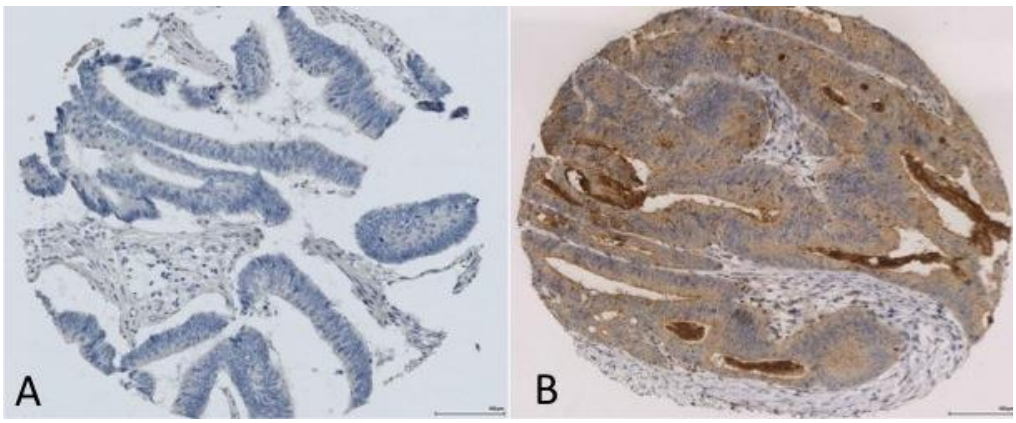


Figure 5.11: FAK (tyr 861) Immunohistochemistry

Representative areas of cancer cells showing high and low FAK (tyr 861) expression (A and B

5.3.3.1 Cytoplasmic FAK (tyr 861) expression

Figure 5.12 demonstrates that cytoplasmic FAK (tyr 861) expression does not follow any particular distribution (histogram, figure 5.12) and all measurements ≥0 are considered outliers (boxplot, figure 5.12). The measurements ranged from 0.00 – 60.00 and had a median of 0 (IQR +/- 0.00).

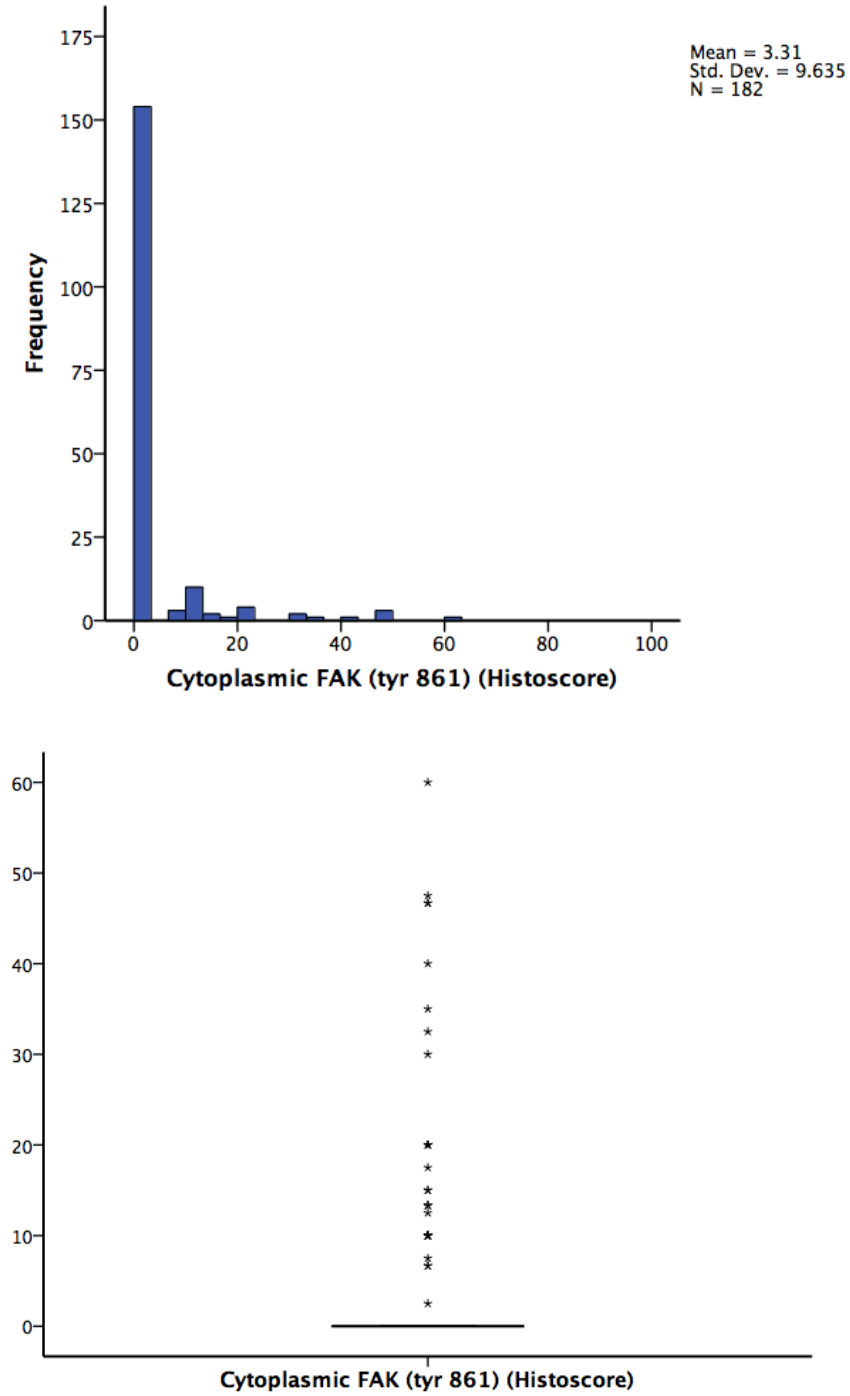
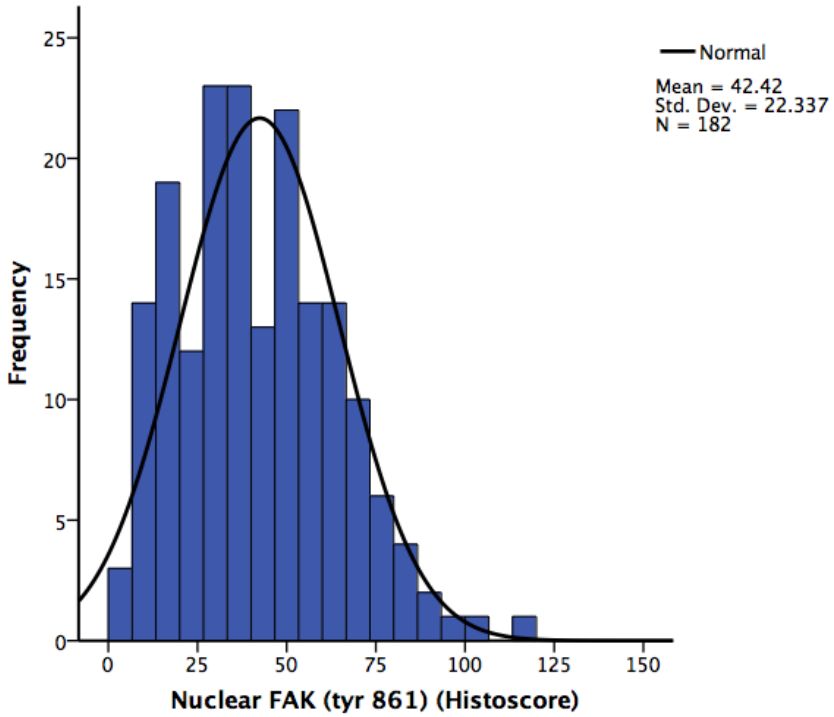


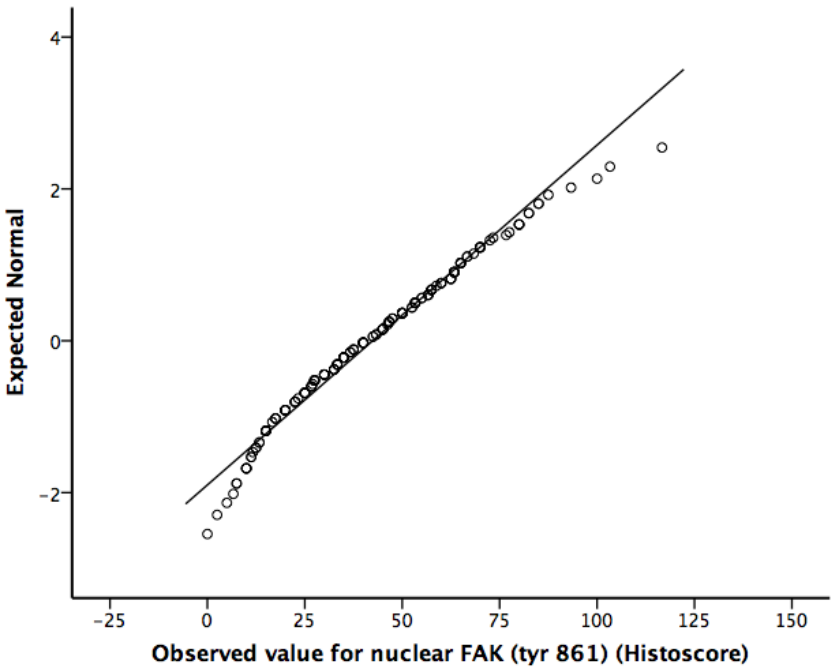
Figure 5.12: Distribution of measurements for cytoplasmic FAK (tyr 861)

Histogram demonstrating the distribution of cytoplasmic FAK (tyr 861) measurements. Boxplot demonstrating the median measurement and outliers of cytoplasmic FAK (tyr 861).

5.3.3.2 Nuclear FAK (tyr 861) expression

Figure 5.13 demonstrates that nuclear FAK (tyr 861) expression does not follow a precise normal distribution (histogram, figure 5.13), which is supported by a Shapiro-Wilk statistic of 0.978, df 182, p=0.006. The measurements ranged from 0.00 – 116.67 and had a median of 40.00 (IQR 7.50 – 72.50).





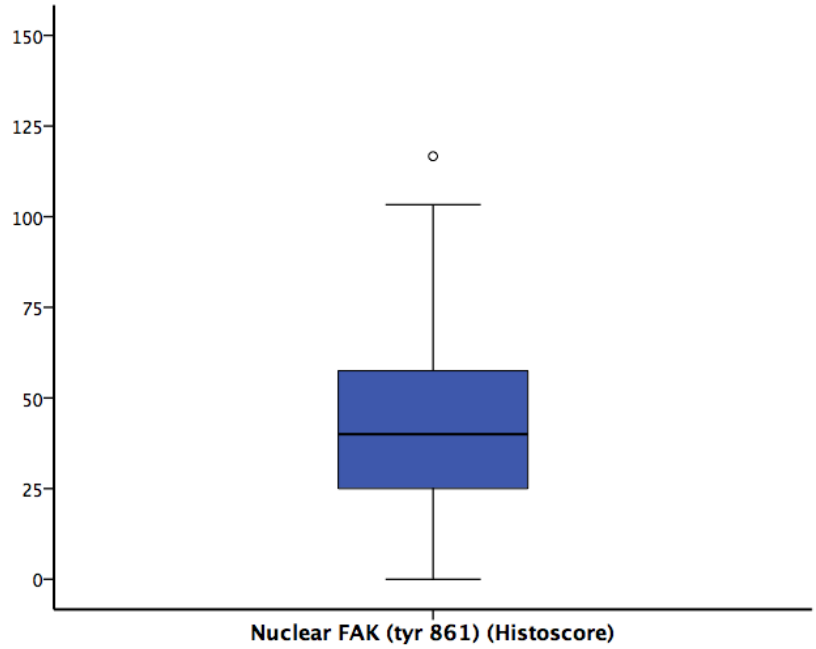


Figure 5.13 Distribution of measurements for nuclear FAK (tyr 861)

Histogram demonstrating the distribution of nuclear FAK (tyr 861). Boxplot demonstrating the median measurement, interquartile range and outliers of nuclear FAK (tyr 861).

5.3.4 Generation of cut offs and association with survival

There are many factors that influence overall survival. Tumour related biomarkers are cancer specific and therefore recurrence and recurrence-free survival has been used to choose optimal cutoffs for dichotomisation.

5.3.4.1 Tumour cancer cell FGR

Membrane FGR

When ROC analysis was performed using recurrence as the endpoint, the AUC was calculated for membranous FGR as a continuous variable, categorised as a dichotomy around the median, tertiles and quartiles. The AUCs were 0.52 (95% CI 0.43 – 0.61, p=0.636) for membranous FGR as a continuous variable, 0.52 (95% CI 0.43 – 0.61, p=0.746) for membranous FGR as a median, 0.52 (95% CI 0.43 – 0.61, p=0.700) for membranous FGR as tertiles and an AUC of 0.52 (95% CI 0.43 – 0.61, p=0.663) for membranous FGR as quartiles (figure 5.14).

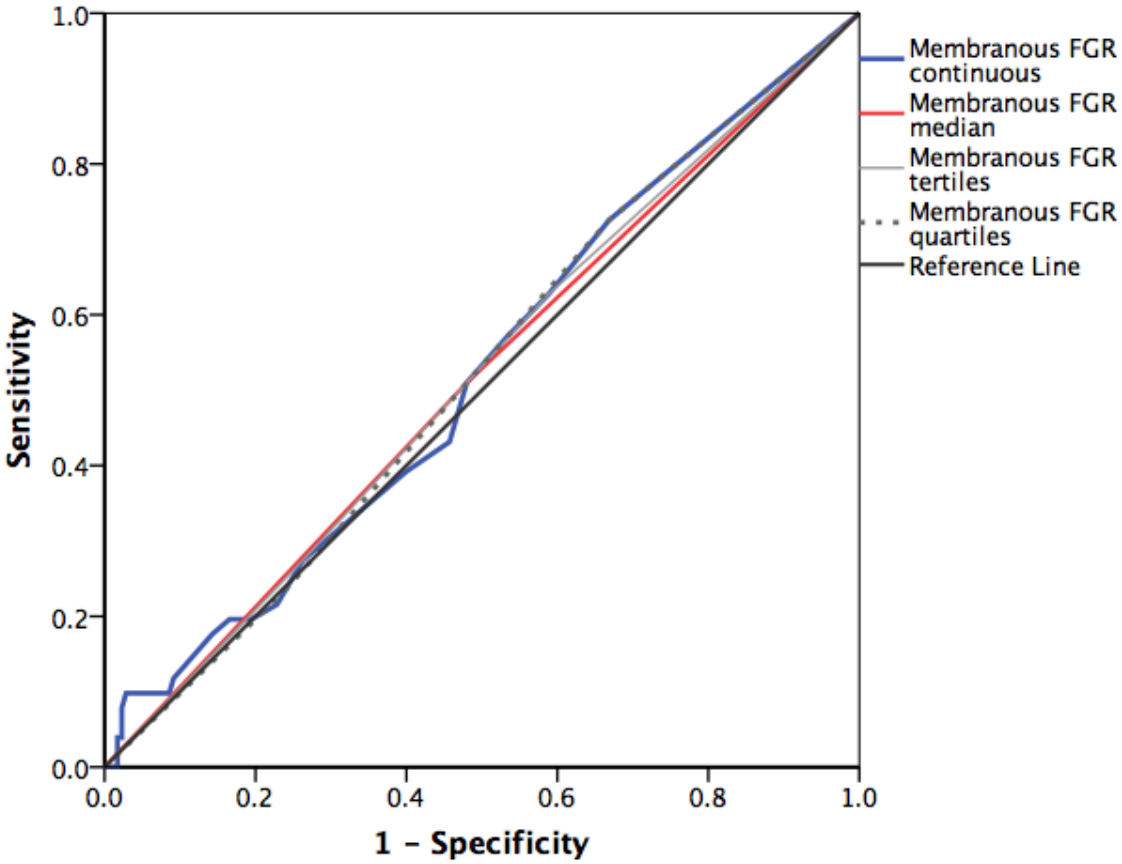
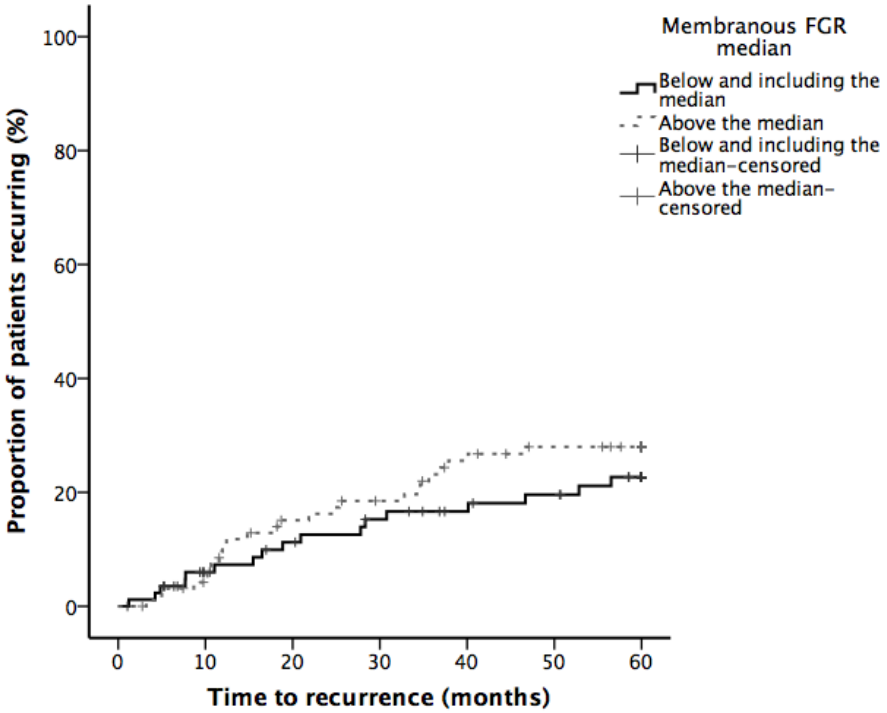
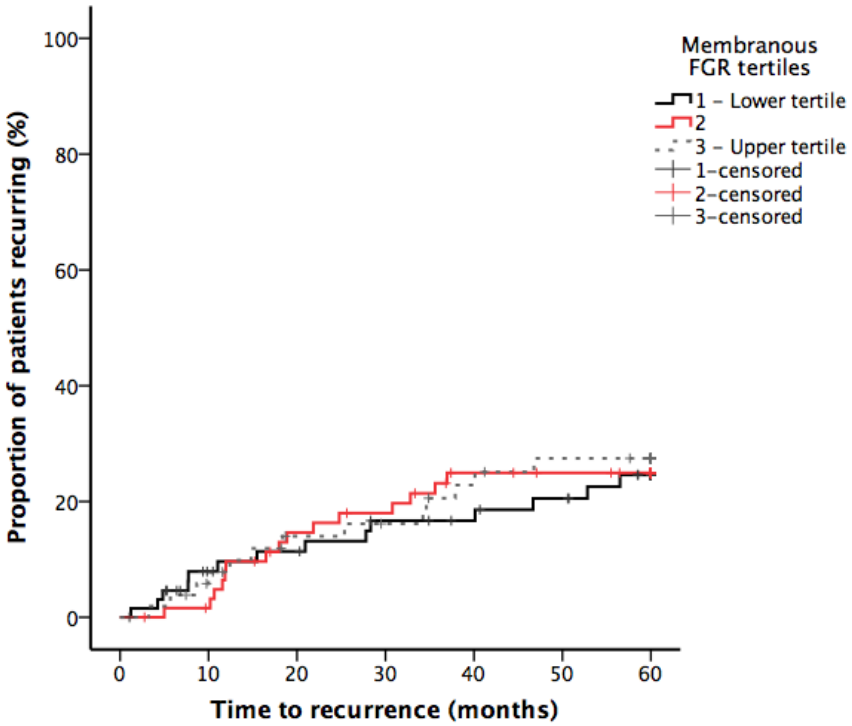


Figure 5.14: The predictive value of membranous FGR in identifying patients who will develop cancer recurrence

Receiver-operator-characteristic curve demonstrating the predictive value of membrane FGR in identifying patients who will develop cancer recurrence.

On Kaplan-Meier analysis, there was no significant trend between membranous FGR expression when categorised around the median (p=0.414), as tertiles (p=0.949), as quartiles (p=0.804) and then dichotomised above the upper quartile (p=0.990) (figure 5.15)


Membranous FGR median
Below and including the median
Above the median
Below and including the median-censored
Above the median-censored
Proportion of patients recurring (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Time to recurrence (months)


Membranous FGR tertiles
1 - Lower tertile
2
3 - Upper tertile
1-censored
2-censored
3-censored
Proportion of patients recurring (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Time to recurrence (months)

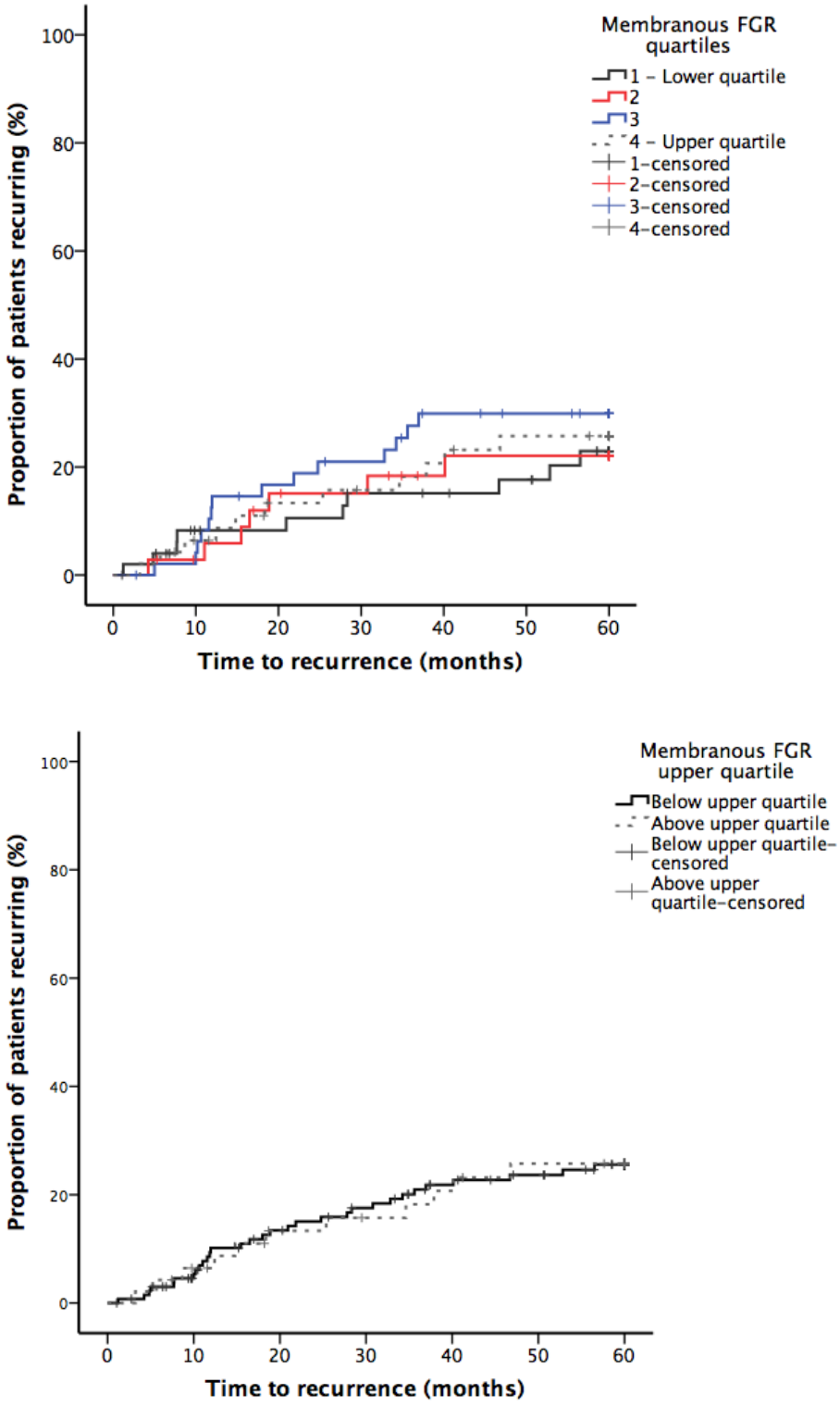


Figure 5.15: Kaplan-Meier curves demonstrating the association between membranous FGR expression and recurrence-free survival in patients with colorectal cancer

Cytoplasmic FGR

When ROC analysis was performed using recurrence as the endpoint, the AUC was calculated for cytoplasmic FGR as a continuous variable, and then categorised as a

dichotomy around the median, tertiles and quartiles. The AUCs were 0.43 (95% CI 0.35 – 0.52, p=0.134) for cytoplasmic FGR as a continuous variable, 0.47 (95% CI 0.38 – 0.56, p=0.532) for cytoplasmic FGR as a median, 0.43 (95% CI 0.34 – 0.52, p=0.130) for cytoplasmic FGR as tertiles and an AUC of 0.43 (95% CI 0.35 – 0.52, p=0.137) for cytoplasmic FGR as quartiles (figure 5.16). Furthermore, on review of the ROC curve, cytoplasmic FGR as a quartile closely follows that of continuous cytoplasmic FGR.

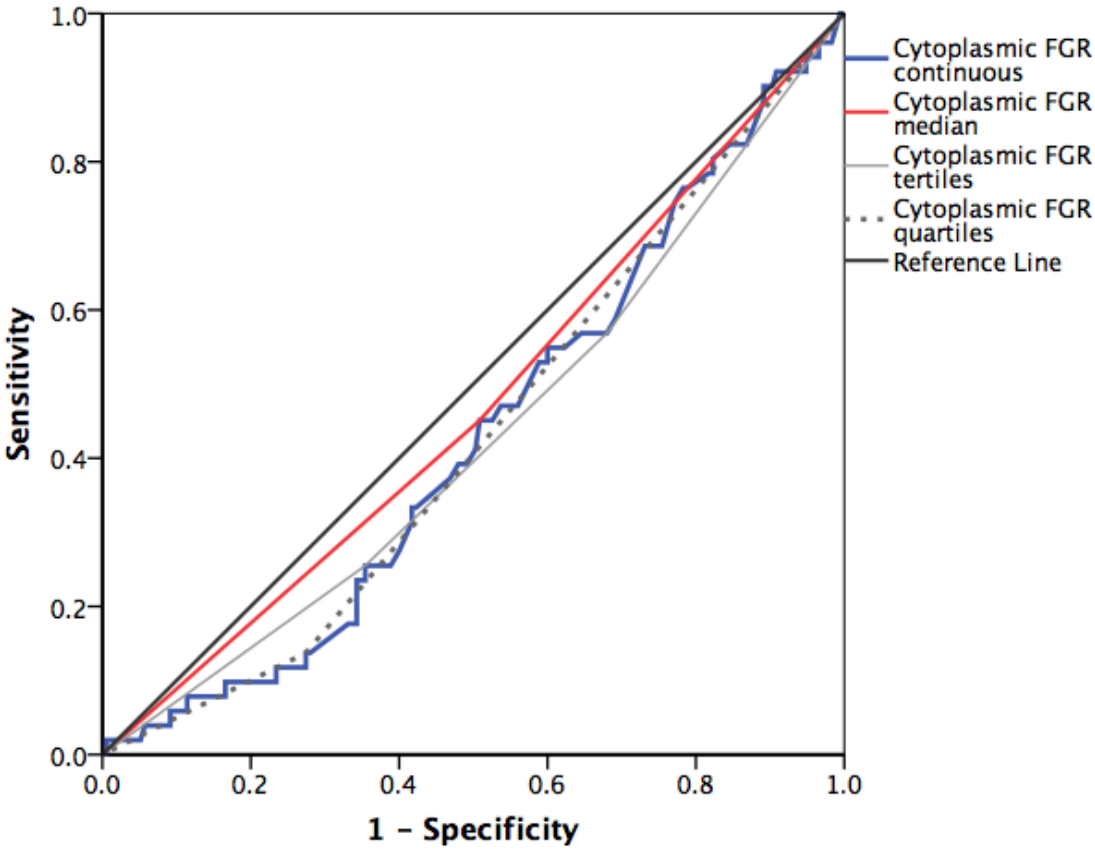
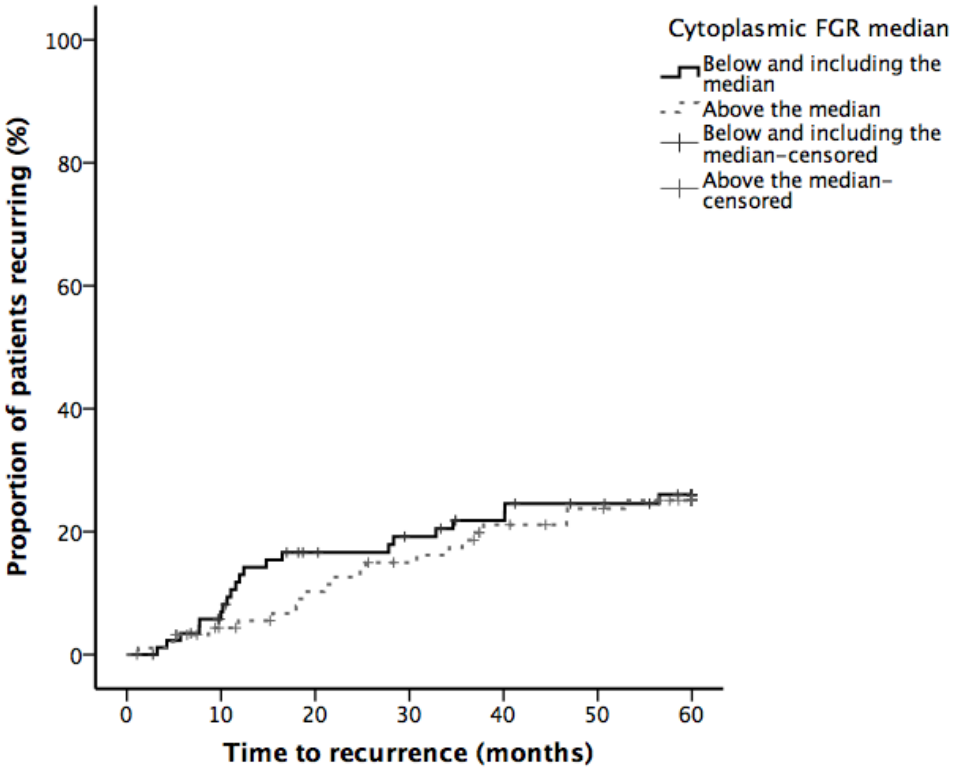
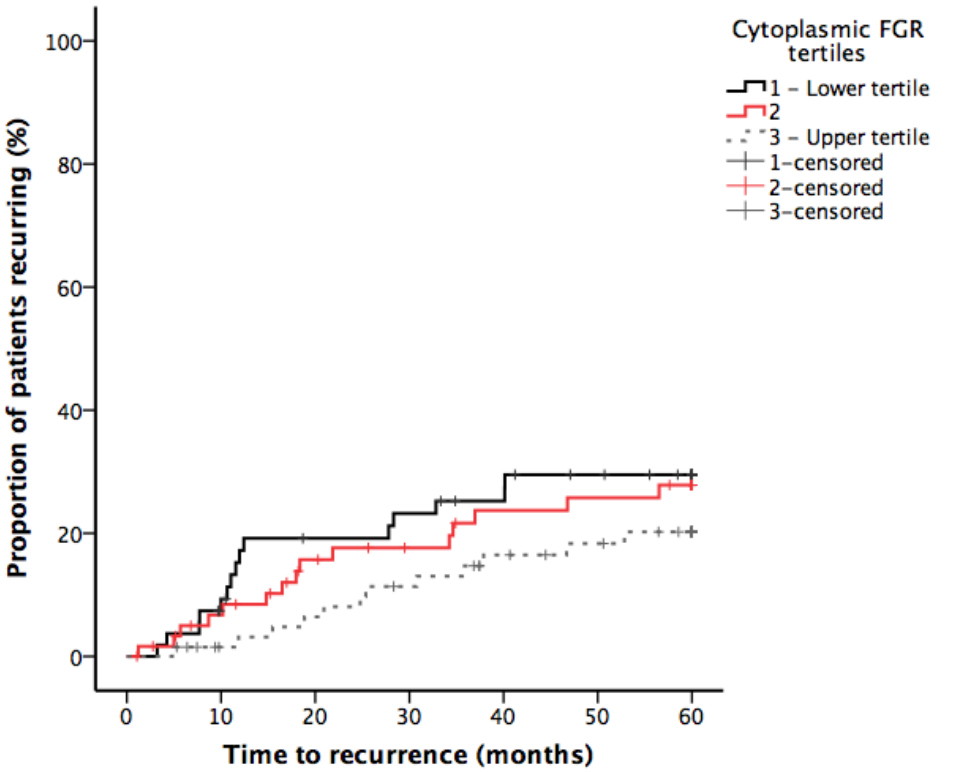


Figure 5.16: The predictive value of cytoplasm FGR in identifying patients who will develop cancer recurrence

Receiver-operator-characteristic curve demonstrating the predictive value of cytoplasmic FGR in identifying patients who will develop cancer recurrence.

On Kaplan-Meier analysis, there was no significant trend between membranous FGR expression when categorised around the median (p=0.740), as tertiles (p=0.367) and quartiles (p=0.103), however, when dichotomised above the upper quartile cytoplasmic FGR was associated with improved survival (p=0.041) (figure 5.17).


Cytoplasmic FGR median
Below and including the median
Above the median
Below and including the median-censored
Above the median-censored
Proportion of patients recurring (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Time to recurrence (months)


Cytoplasmic FGR tertiles
1 - Lower tertile
2
3 - Upper tertile
1-censored
2-censored
3-censored
Proportion of patients recurring (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Time to recurrence (months)

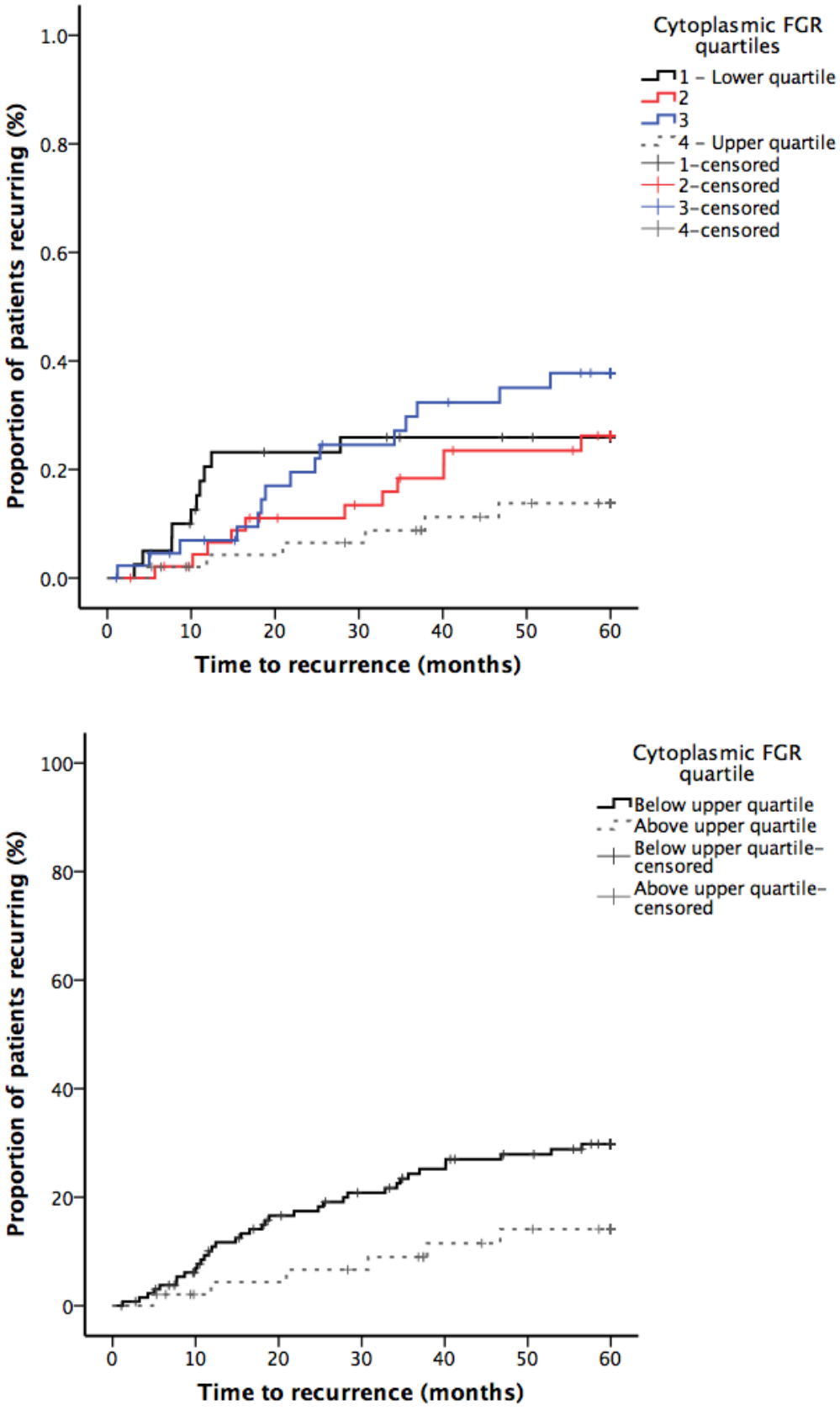


Figure 5.17: Kaplan-Meier curves demonstrating the association between cytoplasmic FGR expression and recurrence-free survival in patients with colorectal cancer

Nuclear FGR

When ROC analysis was performed using recurrence as the endpoint, the AUC was calculated for nuclear FGR as a continuous variable, and then categorised as a dichotomy around the median, tertiles and quartiles. The AUCs were 0.51 (95% CI 0.41 – 0.61, p=0.807) for nuclear FGR as a continuous variable, 0.52 (95% CI 0.42 – 0.62, p=0.640) for nuclear FGR as a median, 0.49 (95% CI 0.39 – 0.59, p=0.857) for nuclear FGR as tertiles and an AUC of 0.48 (95% CI 0.38 – 0.58, p=0.674) for nuclear FGR as quartiles (figure 5.18).

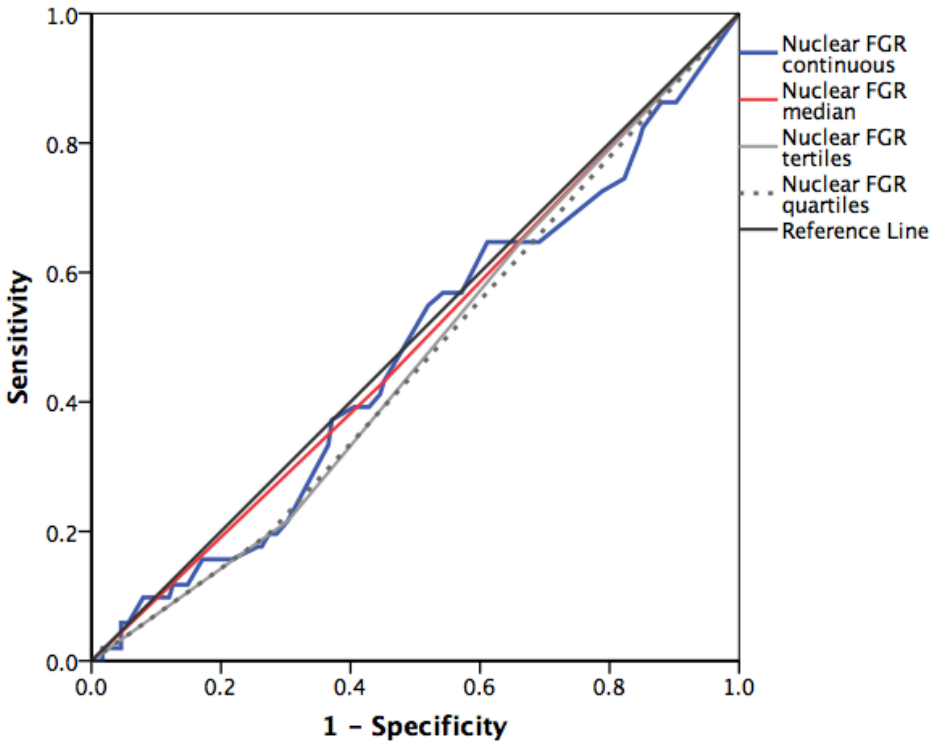
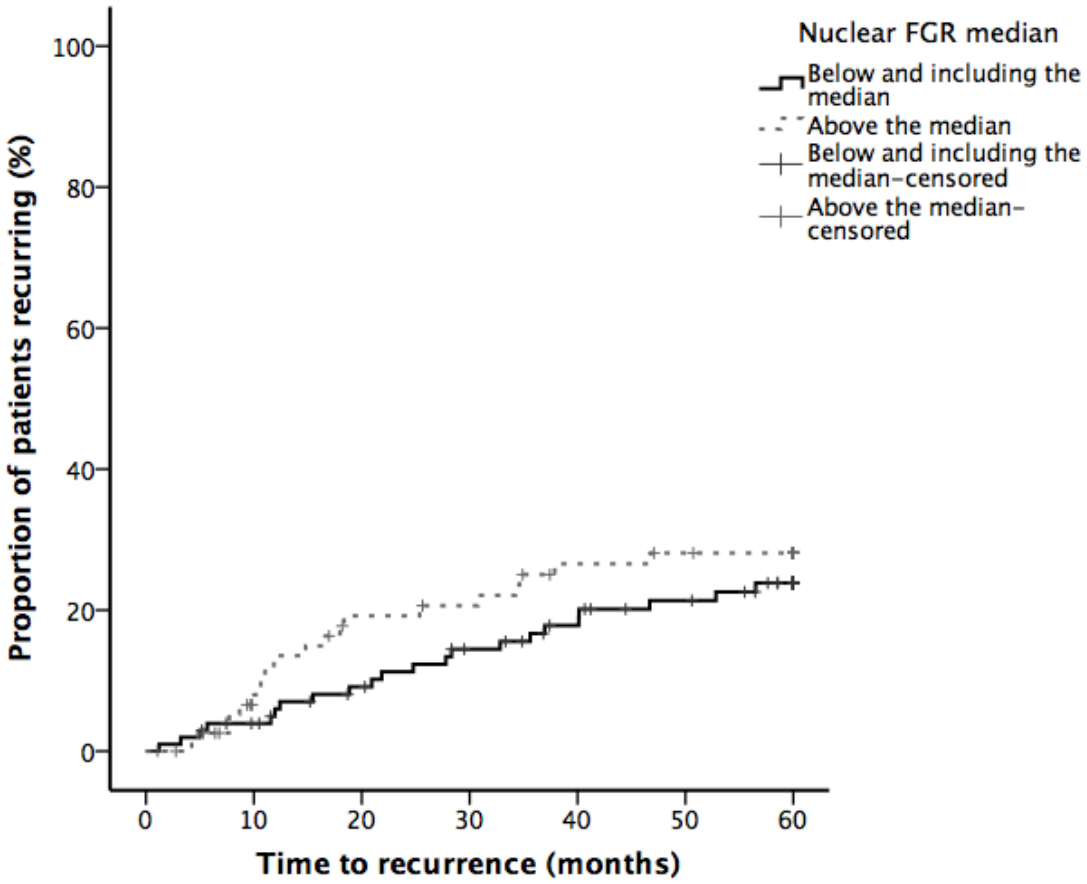
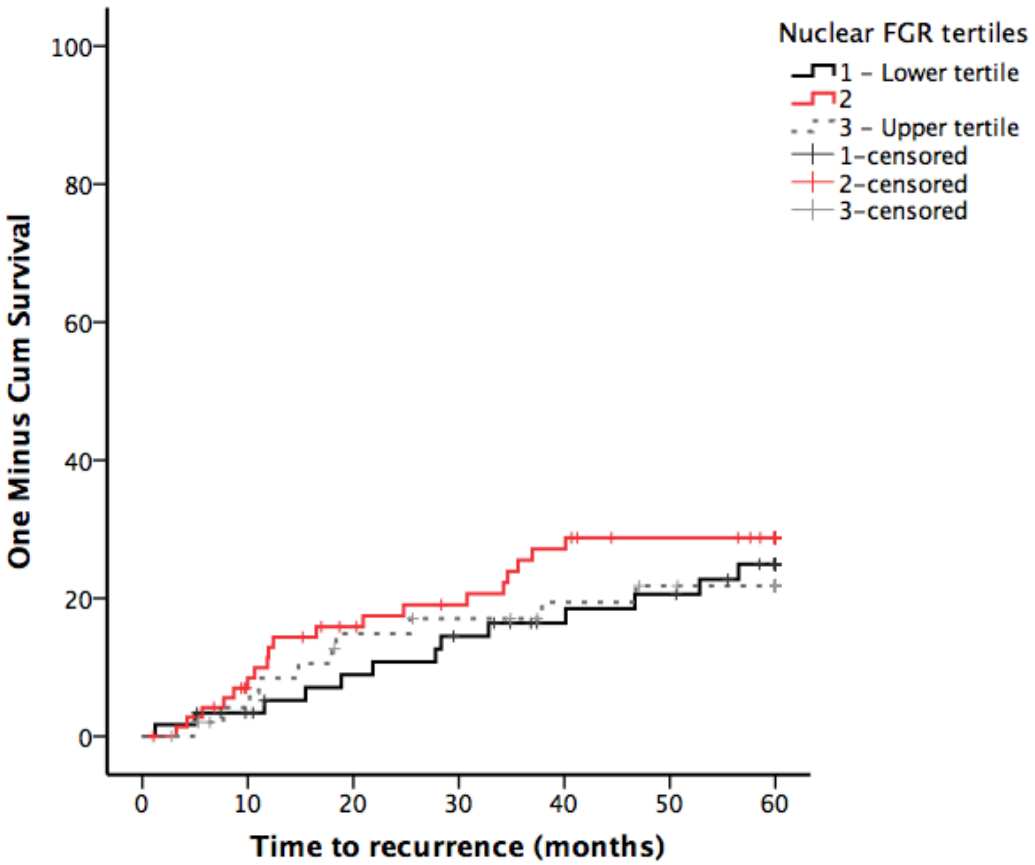


Figure 5.18: The predictive value of nuclear FGR in identifying patients who will develop cancer recurrence

Receiver-operator-characteristic curve demonstrating the predictive value of nuclear FGR in identifying patients who will develop cancer recurrence.

On Kaplan-Meier analysis, there was no significant trend between membranous FGR expression when categorised around the median (p=0.397), as tertiles (p=0.642), as quartiles (p=0.232) and then dichotomised above the upper quartile (p=0.521) (figure 5.19)


Nuclear FGR median
Below and including the median
Above the median
Below and including the median-censored
Above the median-censored
Proportion of patients recurring (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Time to recurrence (months)


Nuclear FGR tertiles
1 - Lower tertile
2
3 - Upper tertile
1-censored
2-censored
3-censored
One Minus Cum Survival
100
80
60
40
20
0
0
10
20
30
40
50
60
Time to recurrence (months)

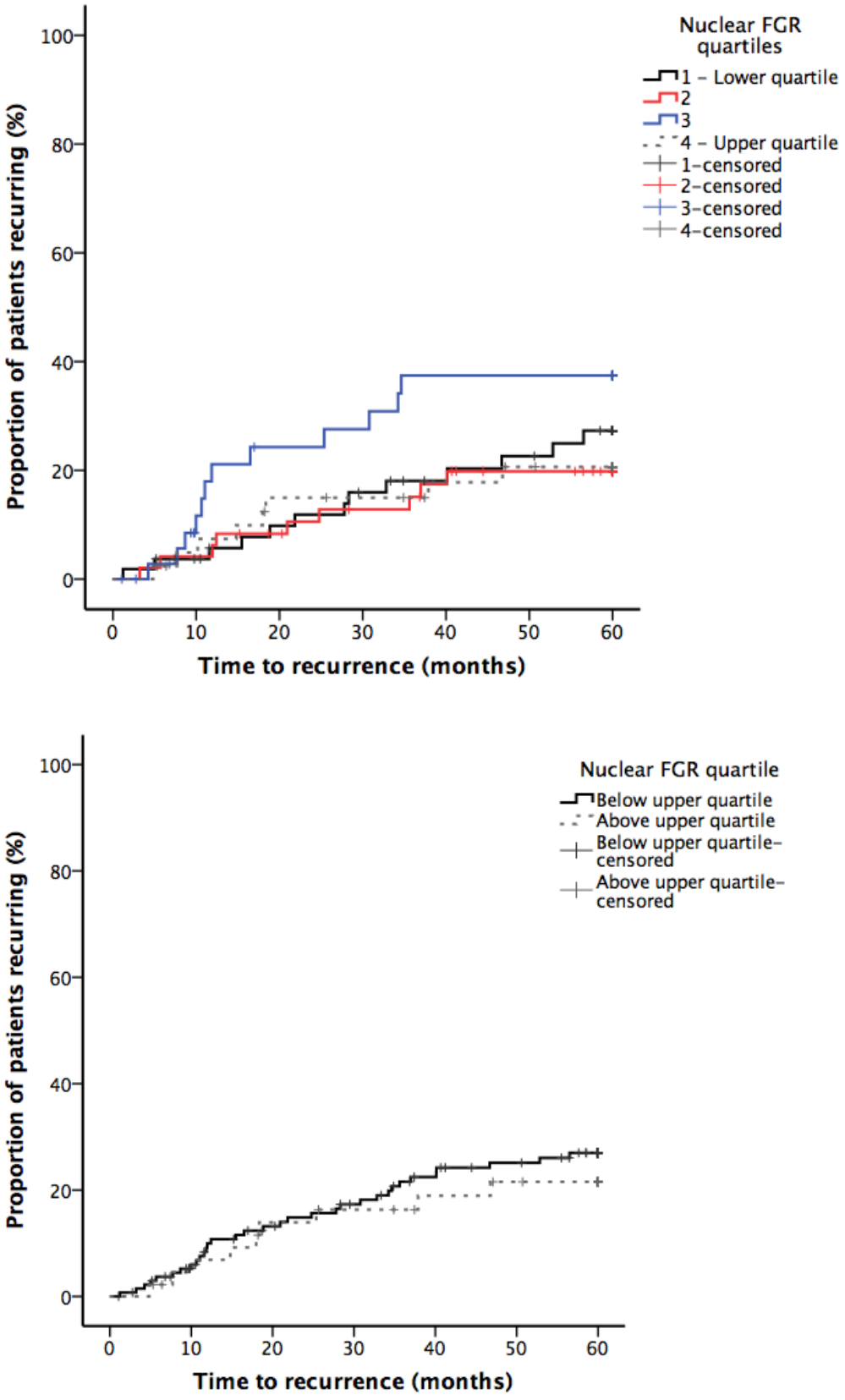


Figure 5.19: Kaplan-Meier curves demonstrating the association between nuclear FGR expression and recurrence-free survival in patients with colorectal cancer

Justification for cancer cell FGR cutoff choice

When FGR was categorised into quartiles, the resultant predictive value and ROC curves offered similar predictive values to FGR as a continuous variable. Kaplan-Meier curves reveal a trend towards significant differences in survival outcomes (p=0.041 for cytoplasmic FGR). Dichotomisation of FGR around the upper quartile has been chosen for validation in cohort 2.

5.3.4.2 Tumour cancer cell HCK

Membraneous HCK

When ROC analysis was performed using recurrence as the endpoint, the AUC was calculated for membranous HCK as a continuous variable, and then categorised as a dichotomy around the median, tertiles and quartiles. The AUCs were 0.51 (95% CI 0.42 – 0.61, p=0.828) for membranous HCK as a continuous variable, 0.54 (95% CI 0.44 – 0.64, p=0.483) for membranous HCK as a median, 0.53 (95% CI 0.43 – 0.62, p=0.623) for membranous HCK as tertiles and an AUC of 0.50 (95% CI 0.41 – 0.60, p=0.960) for membranous HCK as quartiles (figure 5.20).

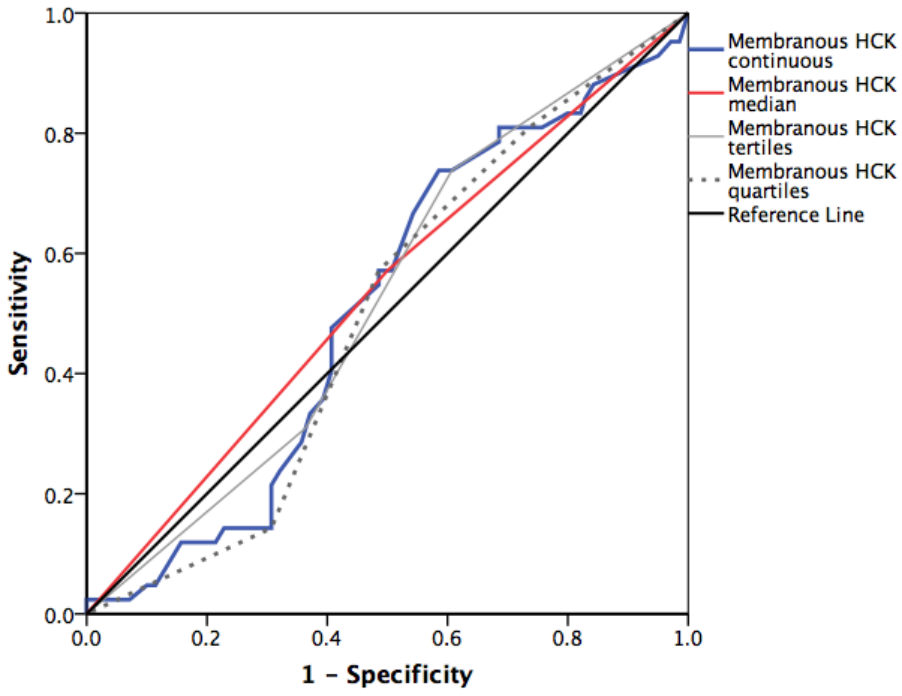
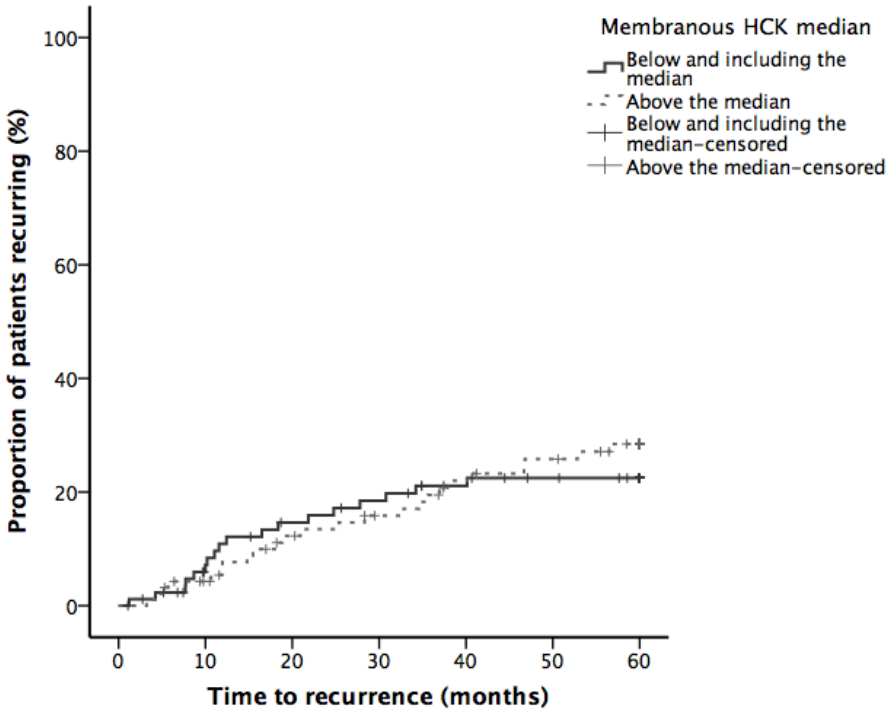
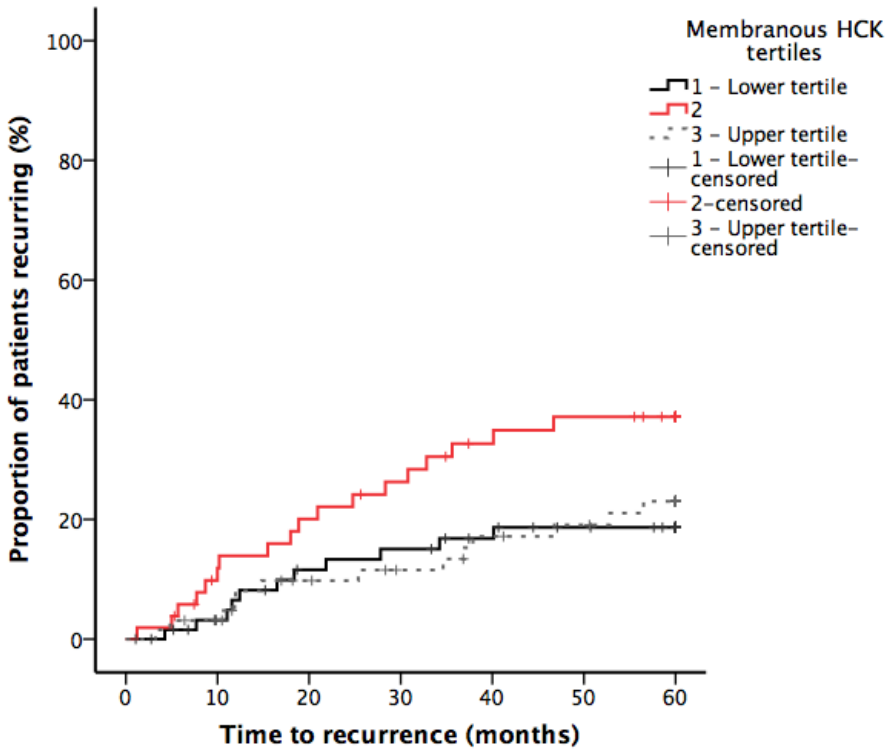


Figure 5.20: The predictive value of membranous HCK in identifying patients who will develop cancer recurrence

Receiver-operator-characteristic curve demonstrating the predictive value of membrane HCK in identifying patients who will develop cancer recurrence.

On Kaplan-Meier analysis, there was no significant trend between membranous HCK expression when categorised around the median (p=0.564), as tertiles (p=0.062), as quartiles (p=0.120) and then dichotomised above the upper quartile (p=0.041) (figure 5.21)





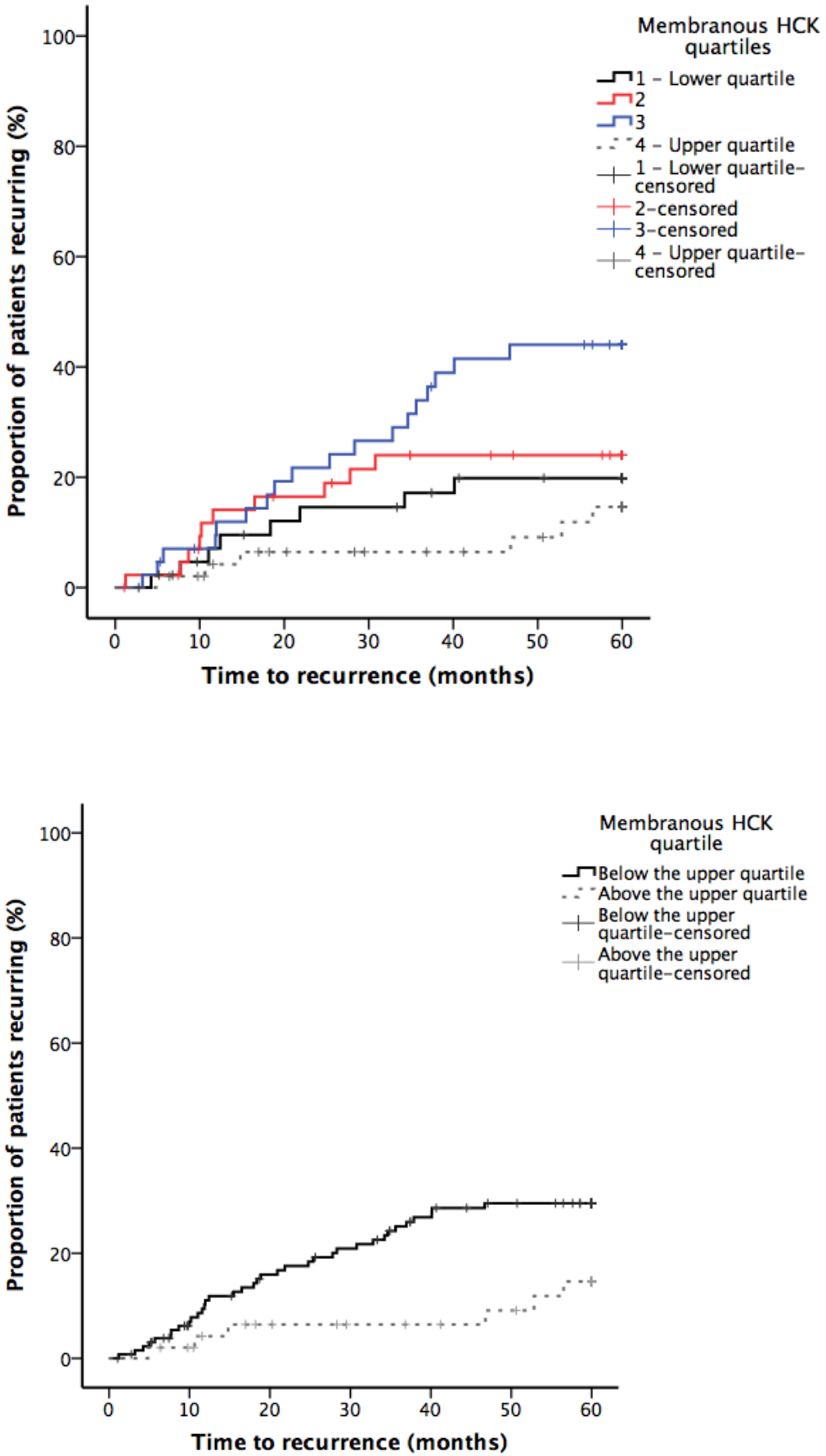


Figure 5.21: Kaplan-Meier curves demonstrating the association between membranous HCK expression and recurrence-free survival in patients with colorectal cancer

Cytoplasmic HCK

When ROC analysis was performed using recurrence as the endpoint, the AUC was calculated for cytoplasmic HCK as a continuous variable, and then categorised as a dichotomy around the median, tertiles and quartiles. The AUCs were 0.56 (95% CI 0.47 – 0.66, p=0.214) for cytoplasmic HCK as a continuous variable, 0.61 (95% CI 0.52 – 0.71, p=0.026) for cytoplasmic HCK as a median, 0.58 (95% CI 0.48 – 0.67, p=0.139) for cytoplasmic HCK as tertiles and an AUC of 0.58 (95% CI 0.48 – 0.67, p=0.124) for cytoplasmic HCK as quartiles (figure 5.22). Furthermore, on review of the ROC curve, cytoplasmic HCK as a quartile closely follows that of continuous cytoplasmic HCK.

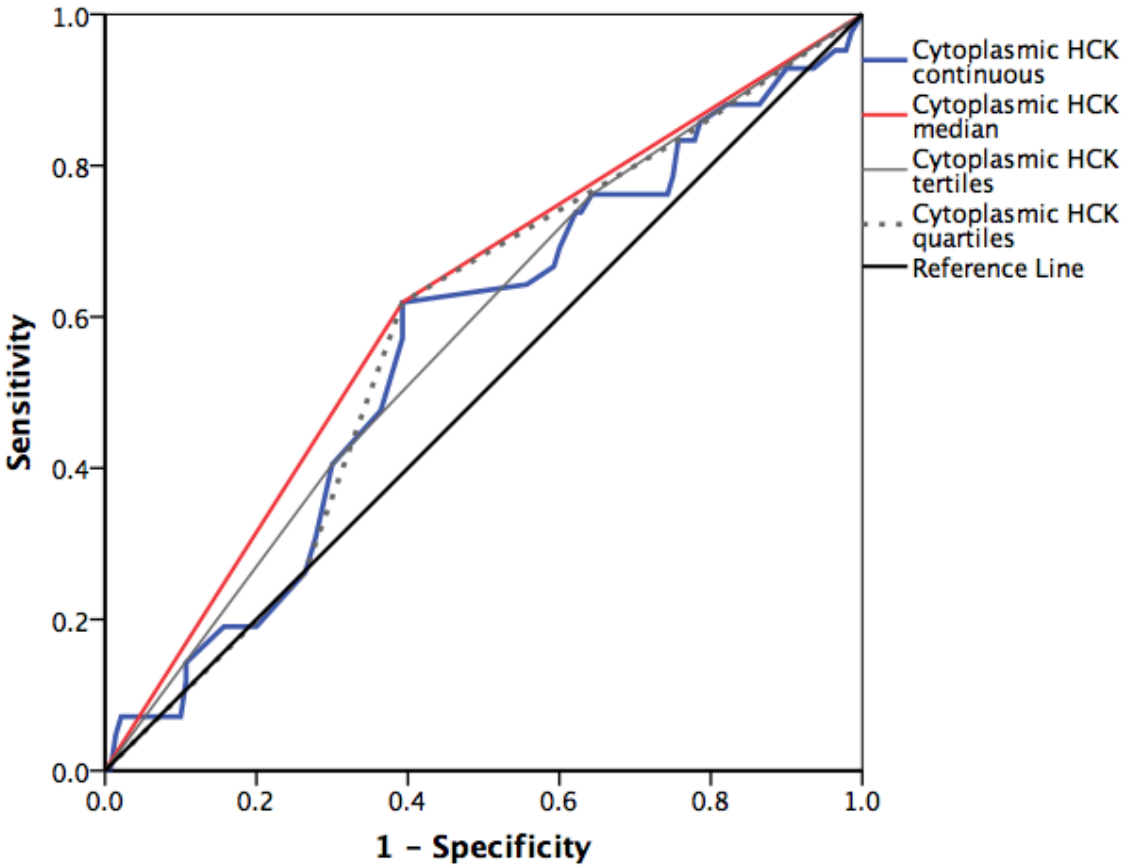
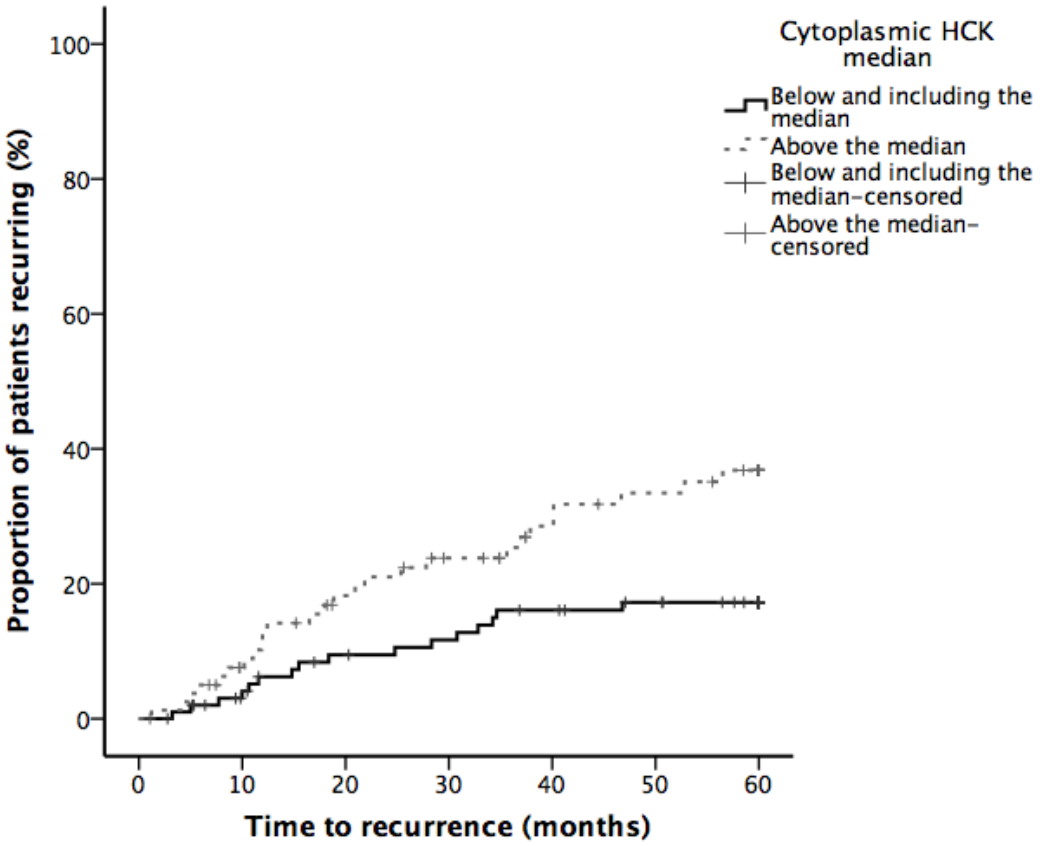
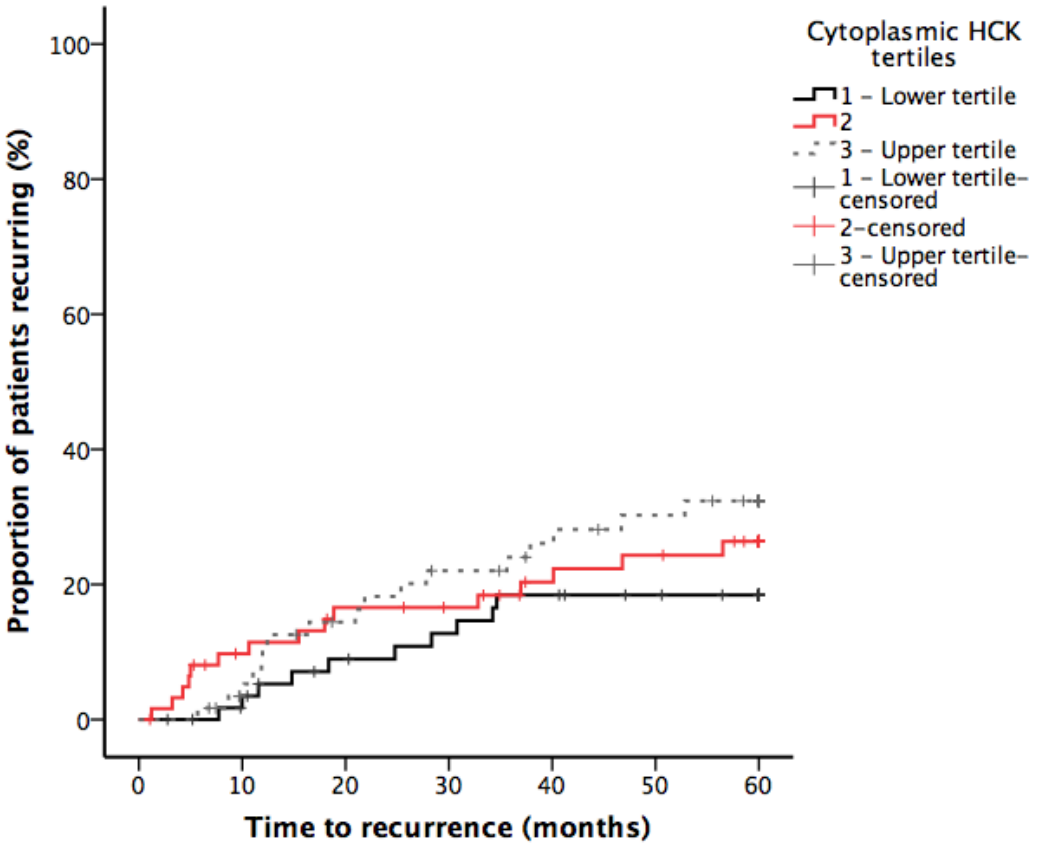


Figure 5.22: The predictive value of cytoplasm HCK in identifying patients who will develop cancer recurrence

Receiver-operator-characteristic curve demonstrating the predictive value of cytoplasmic HCK in identifying patients who will develop cancer recurrence.

On Kaplan-Meier analysis, there was no significant trend between membranous FGR expression when categorised around the median (p=0.007), as tertiles (p=0.295) and quartiles (p=0.001), however, when dichotomised above the upper quartile cytoplasmic HCK was not associated with survival (p=0.874) (figure 5.23).


Cytoplasmic HCK median
Below and including the median
Above the median
Below and including the median-censored
Above the median-censored
Proportion of patients recurring (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Time to recurrence (months)


Cytoplasmic HCK tertiles
1 - Lower tertile
2
3 - Upper tertile
1 - Lower tertile-censored
2-censored
3 - Upper tertile-censored
Proportion of patients recurring (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Time to recurrence (months)

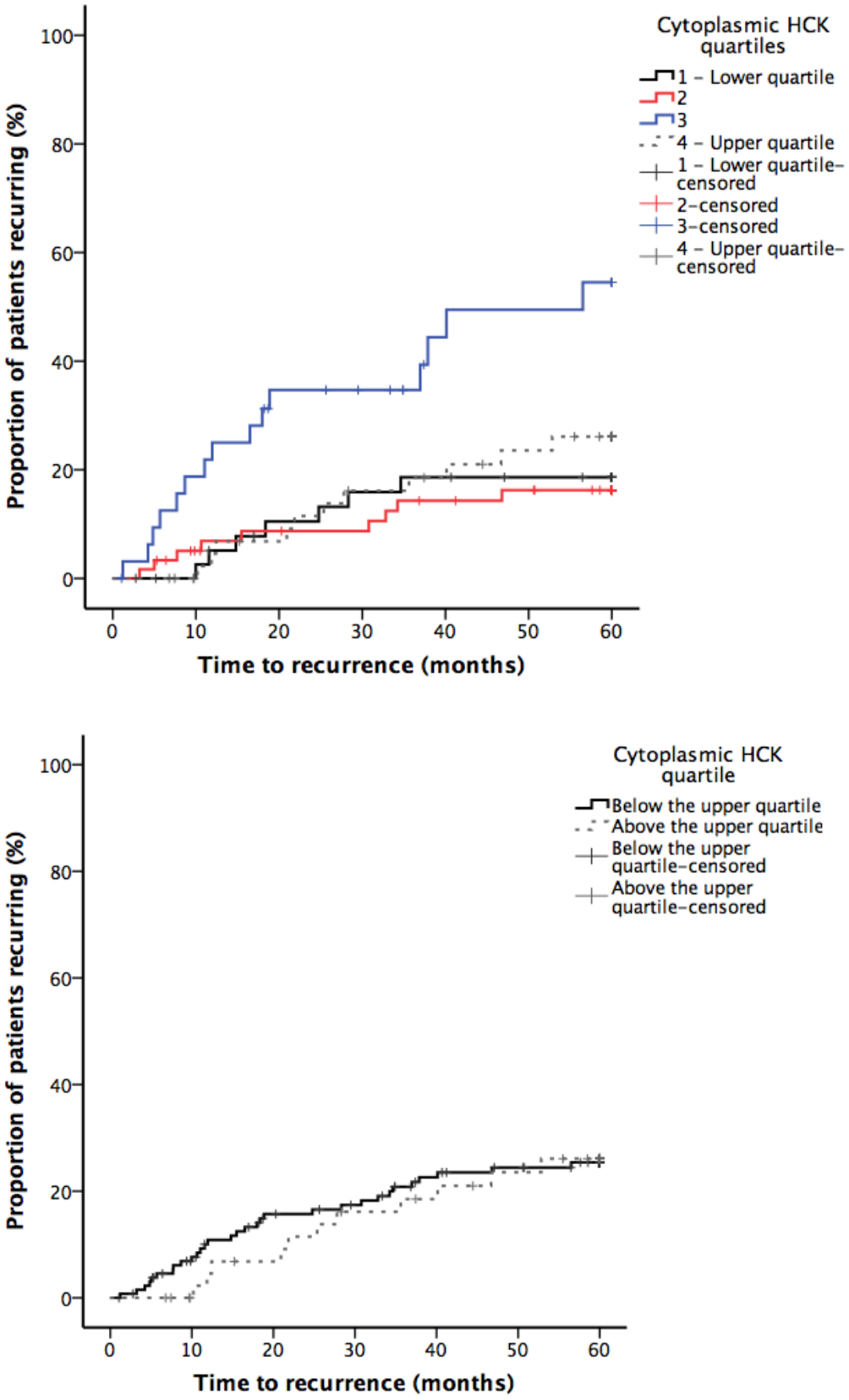


Figure 5.23: Kaplan-Meier curves demonstrating the association between cytoplasmic HCK expression and recurrence-free survival in patients with colorectal cancer

Nuclear HCK

When ROC analysis was performed using recurrence as the endpoint, the AUC was calculated for nuclear HCK as a continuous variable, and then categorised as a dichotomy around the median, tertiles and quartiles. The AUCs were 0.49 (95% CI 0.39 – 0.59, p=0.785) for nuclear HCK as a continuous variable, 0.47 (95% CI 0.37 – 0.57, p=0.498) for nuclear HCK as a median, 0.48 (95% CI 0.38 – 0.58, p=0.708) for nuclear HCK as tertiles and an AUC of 0.47 (95% CI 0.37 – 0.57, p=0.540) for nuclear HCK as quartiles (figure 5.24).

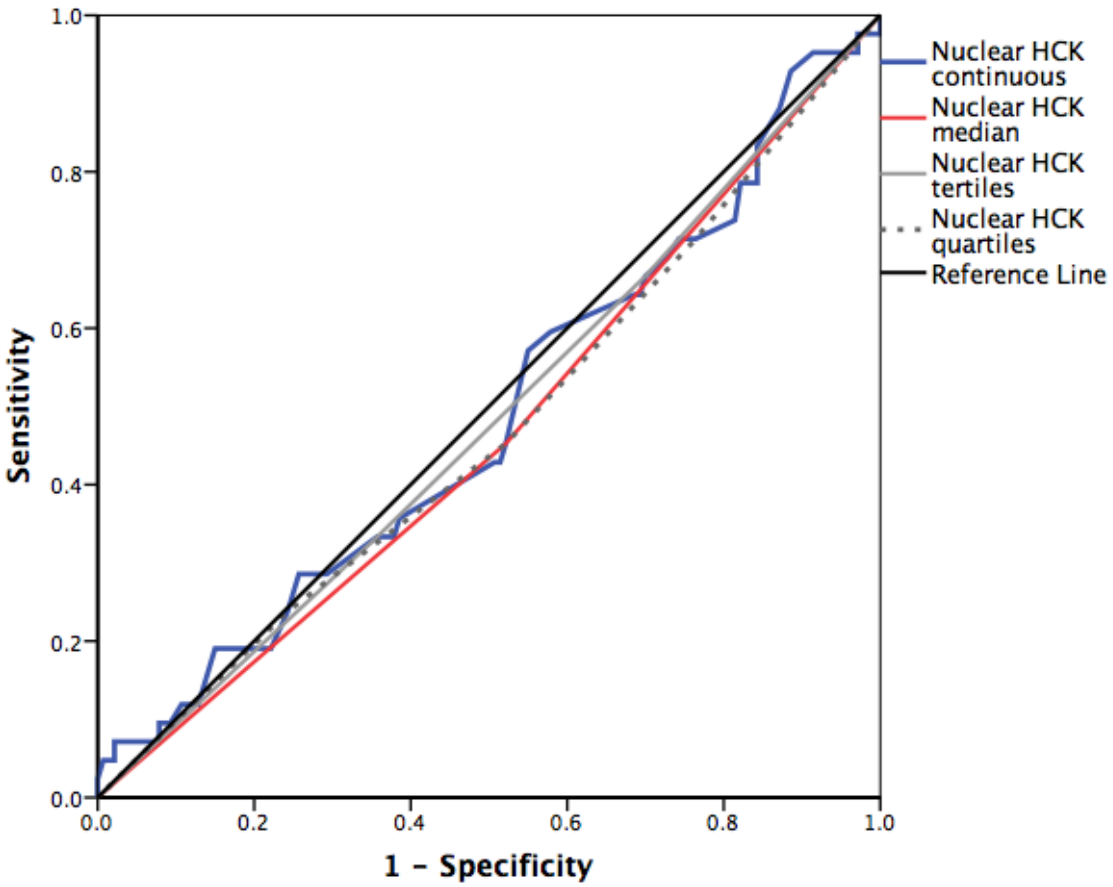
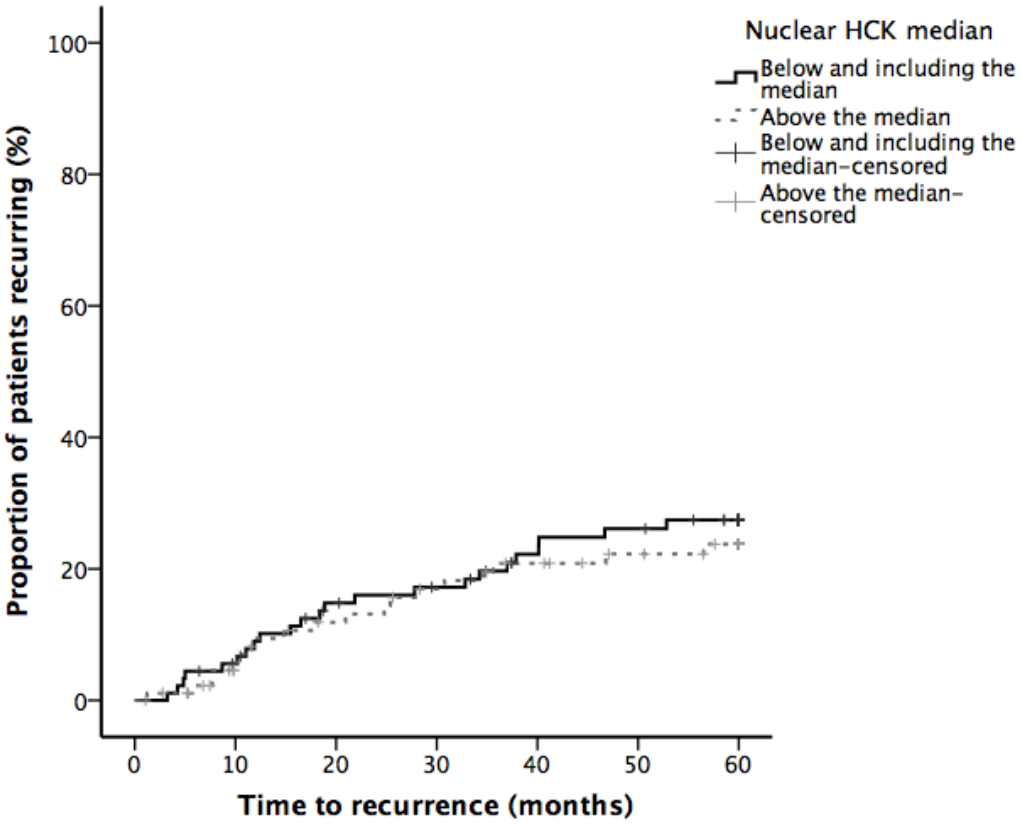
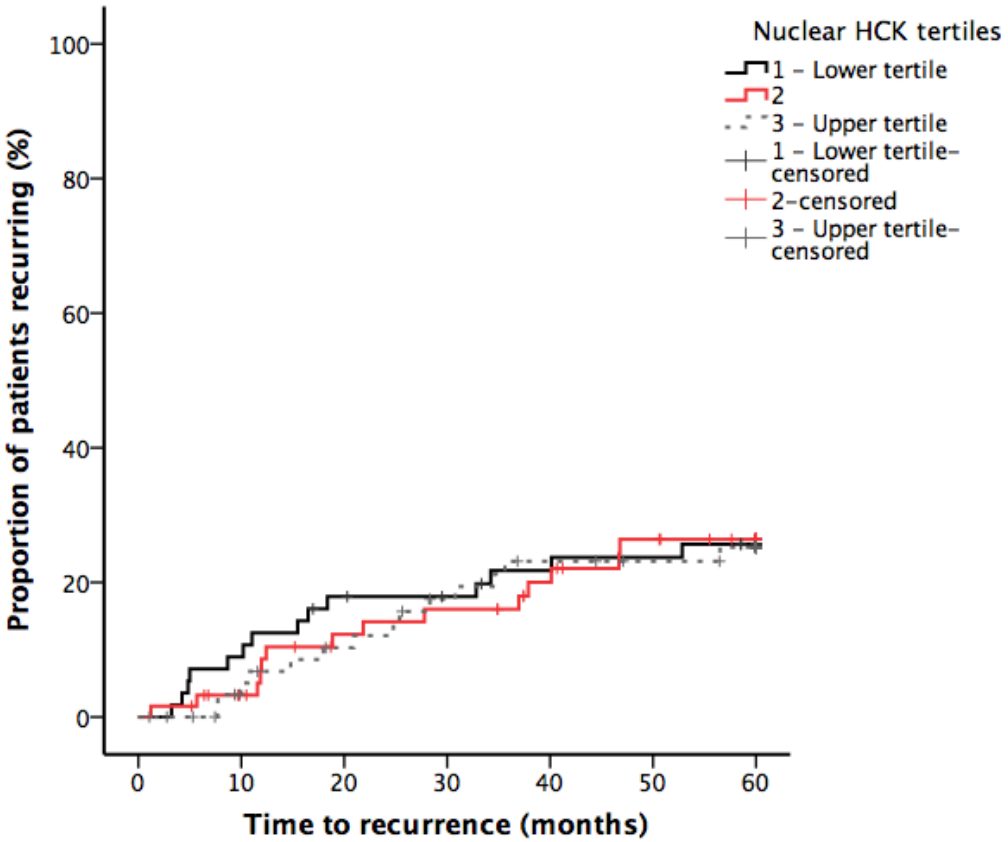


Figure 5.24: The predictive value of nuclear HCK in identifying patients who will develop cancer recurrence

Receiver-operator-characteristic curve demonstrating the predictive value of nuclear HCK in identifying patients who will develop cancer recurrence.

On Kaplan-Meier analysis, there was no significant trend between nuclear HCK expression when categorised around the median (p=0.617), as tertiles (p=0.982), as quartiles (p=0.954) and then dichotomised above the upper quartile (p=0.932) (figure 5.25)


Nuclear HCK median
Below and including the median
Above the median
Below and including the median-censored
Above the median-censored
Proportion of patients recurring (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Time to recurrence (months)


Nuclear HCK tertiles
1 - Lower tertile
2
3 - Upper tertile
1 - Lower tertile-censored
2-censored
3 - Upper tertile-censored
Proportion of patients recurring (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Time to recurrence (months)

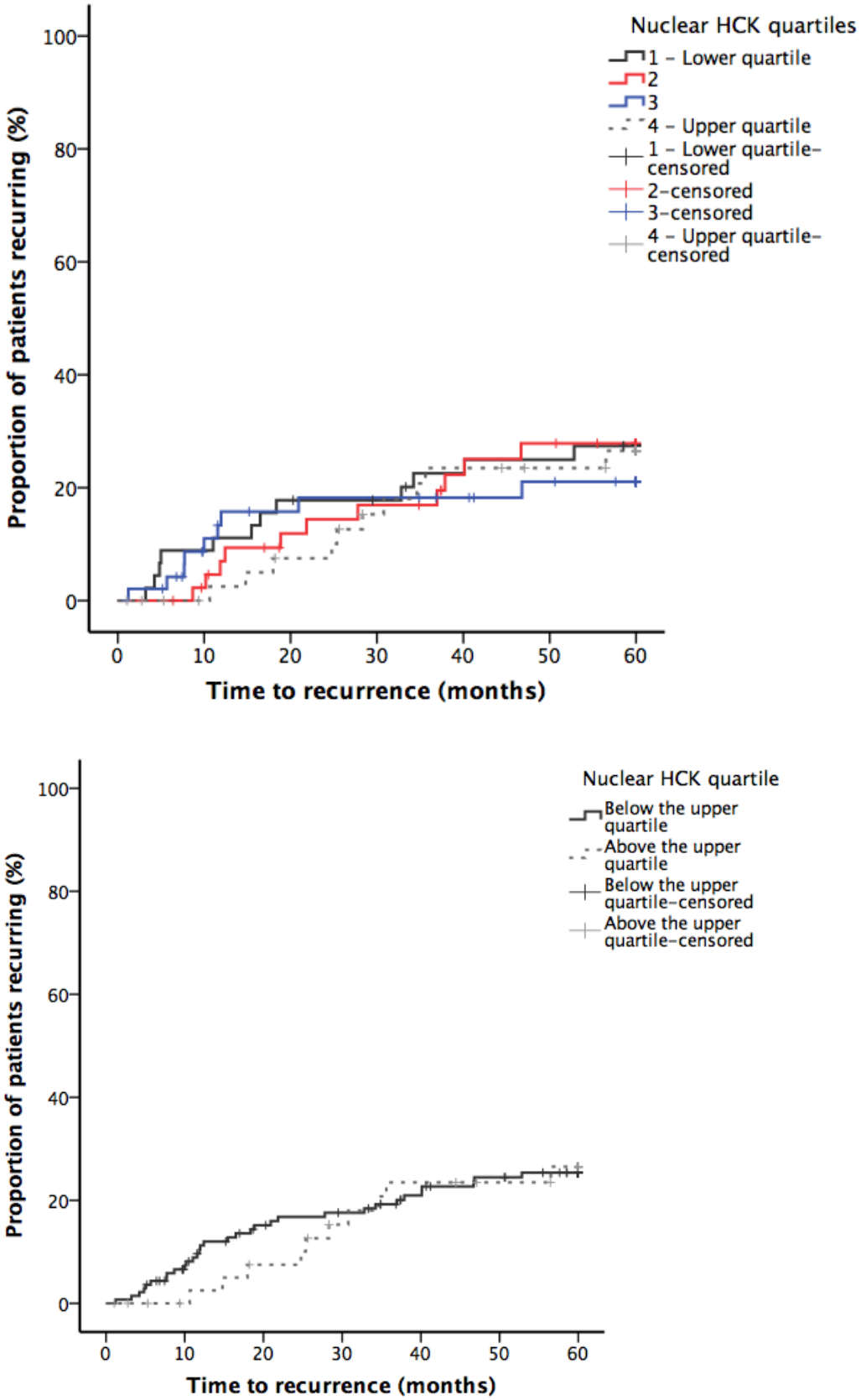


Figure 5.25: Kaplan-Meier curves demonstrating the association between nuclear HCK expression and recurrence-free survival in patients with colorectal cancer

Justification for cancer cell HCK dichotomisation choice

Similar to FGR, categorisation of HCK into quartiles results in a predictive value (AUC) and ROC curve patterns similar to that of HCK as a continuous variable. Again, when reviewing the Kaplan-Meier graphs, the 3rd quartile group appears to have a different outcome when compared to other sub groups, however, identifying this group reliably in a clinical service would be difficult using non-automated histological scoring methods. Theoretically, tumours with the highest expression are likely to exhibit the most adverse behaviour and therefore, categorisation of HCK will be performed around the upper quartile with validation being undertaken in cohort 2.

5.3.4.3 FAK (tyr 861) expression

Cytoplasmic FAK (tyr 861)

When ROC analysis was performed using recurrence as the endpoint, the AUC was calculated for cytoplasmic FAK (tyr 861) as a continuous variable, and then categorised as a dichotomy around the median and upper tertile. The AUCs were 0.50 (95% CI 0.39 – 0.60, p=0.919) for cytoplasmic FAK (tyr 861) as a continuous variable, 0.49 (95% CI 0.39 – 0.59, p=0.833) for cytoplasmic FAK (tyr 861) as a median and 0.53 (95% CI 0.42 – 0.63, p=0.623) for cytoplasmic FAK (tyr 861) as a dichotomy around the upper tertile (figure 5.26)

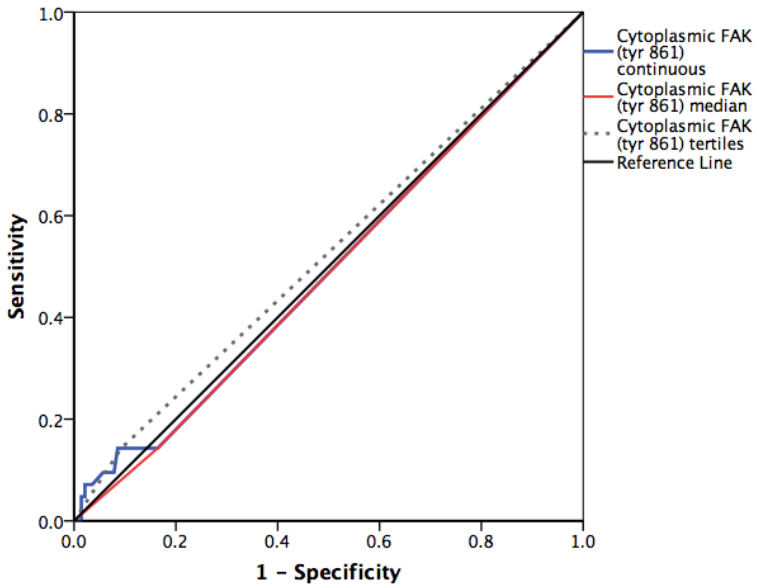


Figure 5.26: The predictive value of cytoplasm FAK (Tyr 861) in identifying patients who will develop cancer recurrence

Receiver-operator-characteristic curve demonstrating the predictive value of cytoplasmic FAK (Tyr 861) *in identifying patients who will develop cancer recurrence.*

On Kaplan-Meier analysis, there was no significant trend between cytoplasmic FAK (tyr 861) expression when categorised around the median (p=0.665) or around the upper tertile (p=0.376) (figure 5.27).

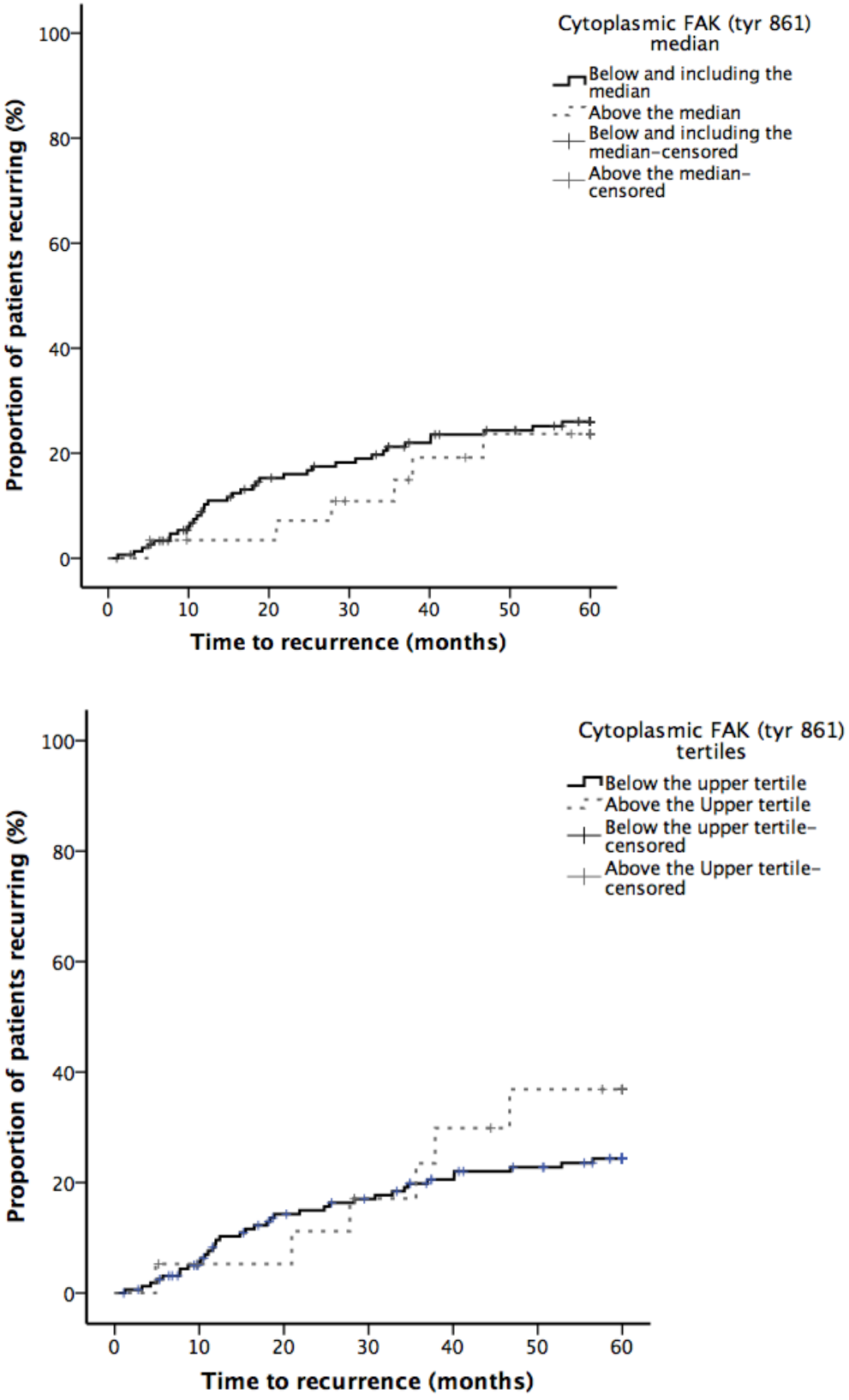


Figure 5.27: Kaplan-Meier curves demonstrating the association between cytoplasmic FAK (tyr 861) expression and recurrence-free survival in patients with colorectal cancer

Nuclear FAK (tyr 861)

When ROC analysis was performed using recurrence as the endpoint, the AUC was calculated for nuclear FAK (tyr 861) as a continuous variable, and then categorised as a dichotomy around the median, tertiles and quartiles. The AUCs were 0.57 (95% CI 0.47 – 0.67, p=0.179) for nuclear FAK (tyr 861) as a continuous variable, 0.59 (95% CI 0.49 – 0.69, p=0.084) for nuclear FAK (tyr 861) as a median and 0.59 (95% CI 0.49 – 0.69, p=0.079) for nuclear FAK (tyr 861) as tertiles (figure 5.28).

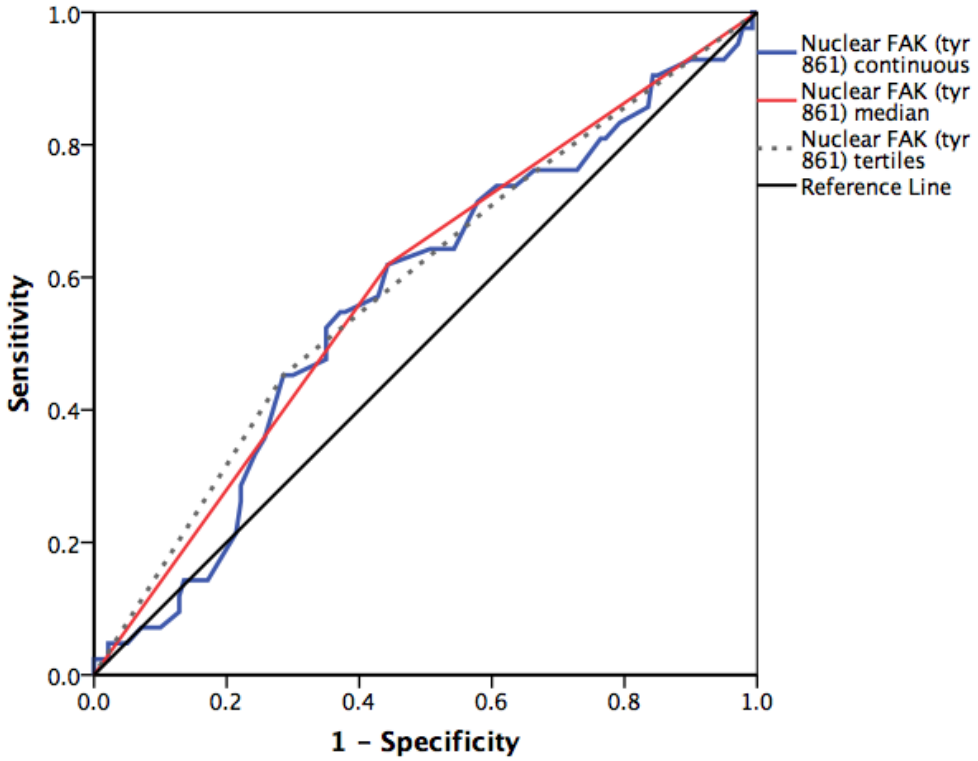
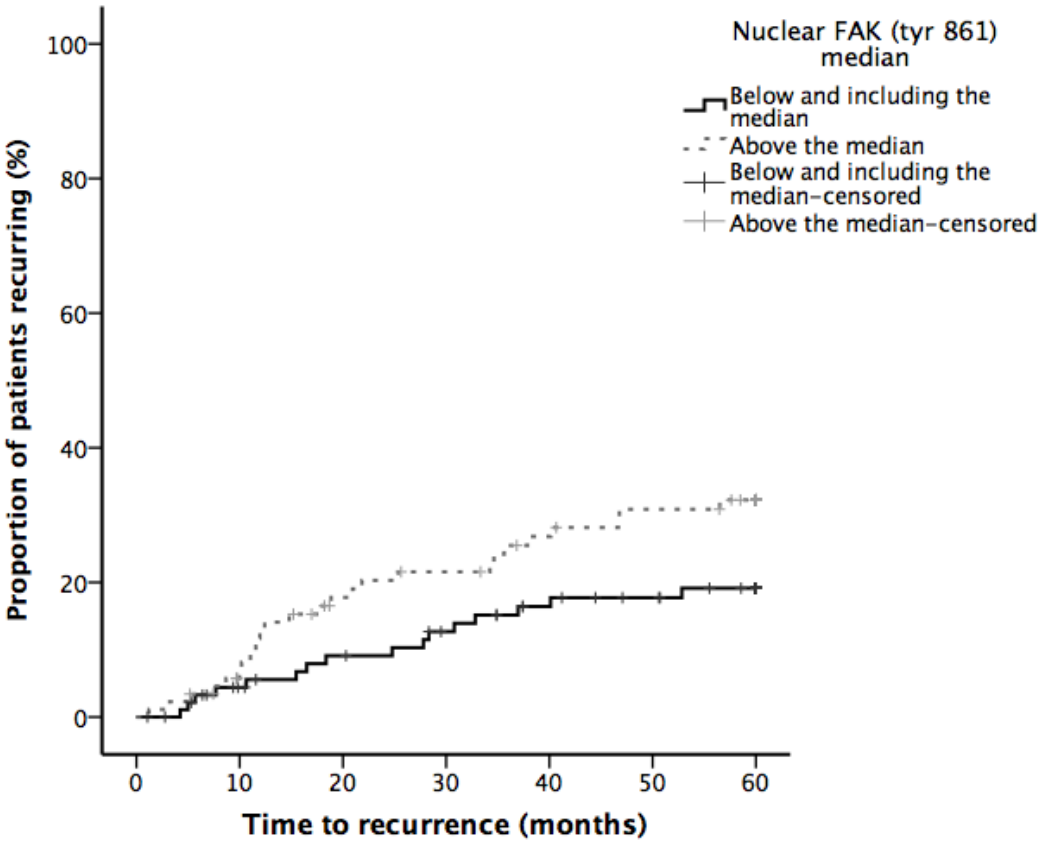
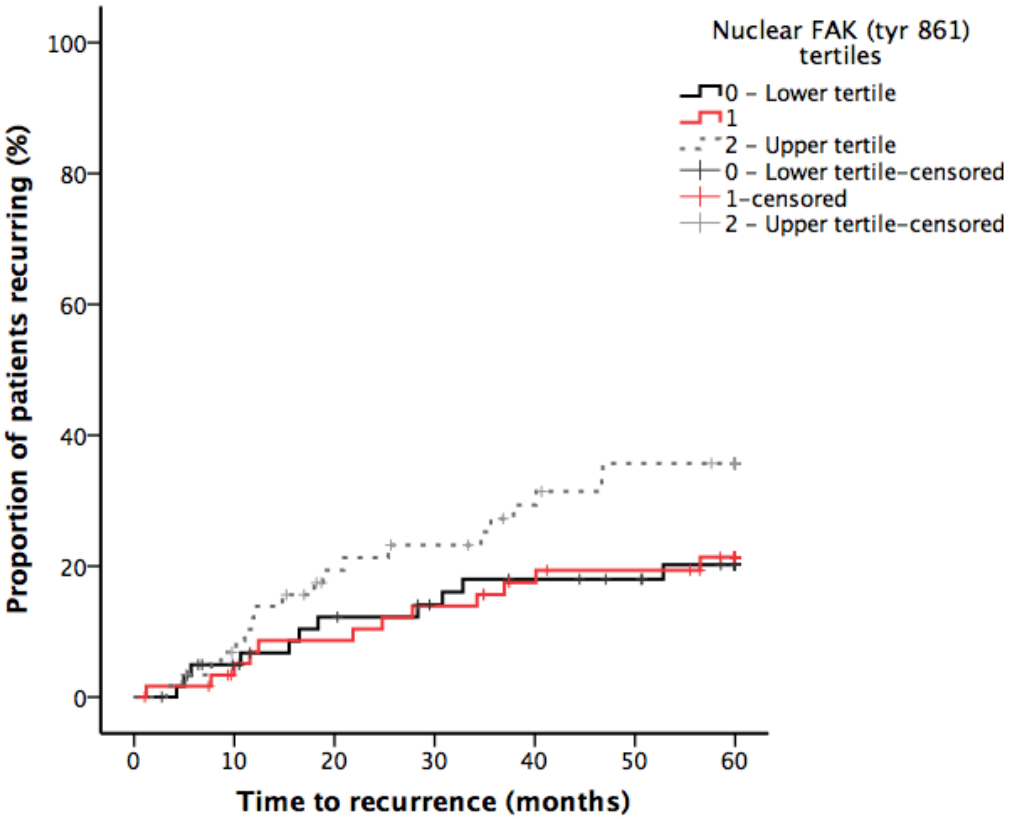


Figure 5.28: The predictive value of nuclear FAK (tyr 861) in identifying patients who will develop cancer recurrence

Receiver-operator-characteristic curve demonstrating the predictive value of nuclear FAK (tyr 861) *in identifying patients who will develop cancer recurrence.*

On Kaplan-Meier analysis, there was no significant trend between nuclear FAK (tyr 861) expression when categorised around the median (p=0.056), as tertiles (p=0.127) and then dichotomised above the upper tertile (p=0.042) (figure 5.29)


Nuclear FAK (tyr 861) median
Below and including the median
Above the median
Below and including the median-censored
Above the median-censored
Proportion of patients recurring (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Time to recurrence (months)


Nuclear FAK (tyr 861) tertiles
0 - Lower tertile
1
2 - Upper tertile
0 - Lower tertile-censored
1-censored
2 - Upper tertile-censored
Proportion of patients recurring (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Time to recurrence (months)

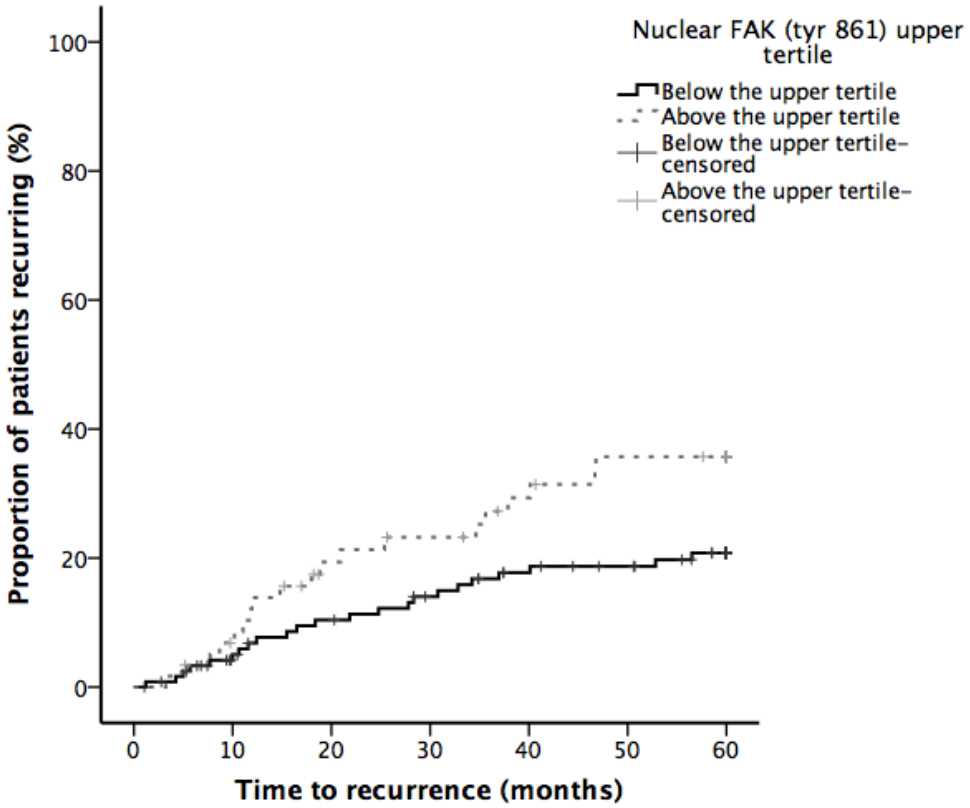


Figure 5.29: Kaplan-Meier curves demonstrating the association between nuclear FAK (tyr 861) expression and recurrence free survival in patients with colorectal cancer

Justification for cancer cell FAK (tyr 861) cutoff choice

Given the low number of tumours expressing cytoplasmic FAK (tyr 861), categorisation into quartiles would not yield reliable results and therefore quartiles have been excluded from the analysis. Categorising FAK (tyr 861) into tertiles results in an AUC and ROC curve patterns similar to that of FAK (tyr 861) as a continuous variable. On reviewing the Kaplan-Meier graphs, the upper tertile group appears to have a different outcome when compared to other sub groups. Categorisation of FAK (tyr 861) will be performed around the upper tertile with validation being undertaken in cohort 2.

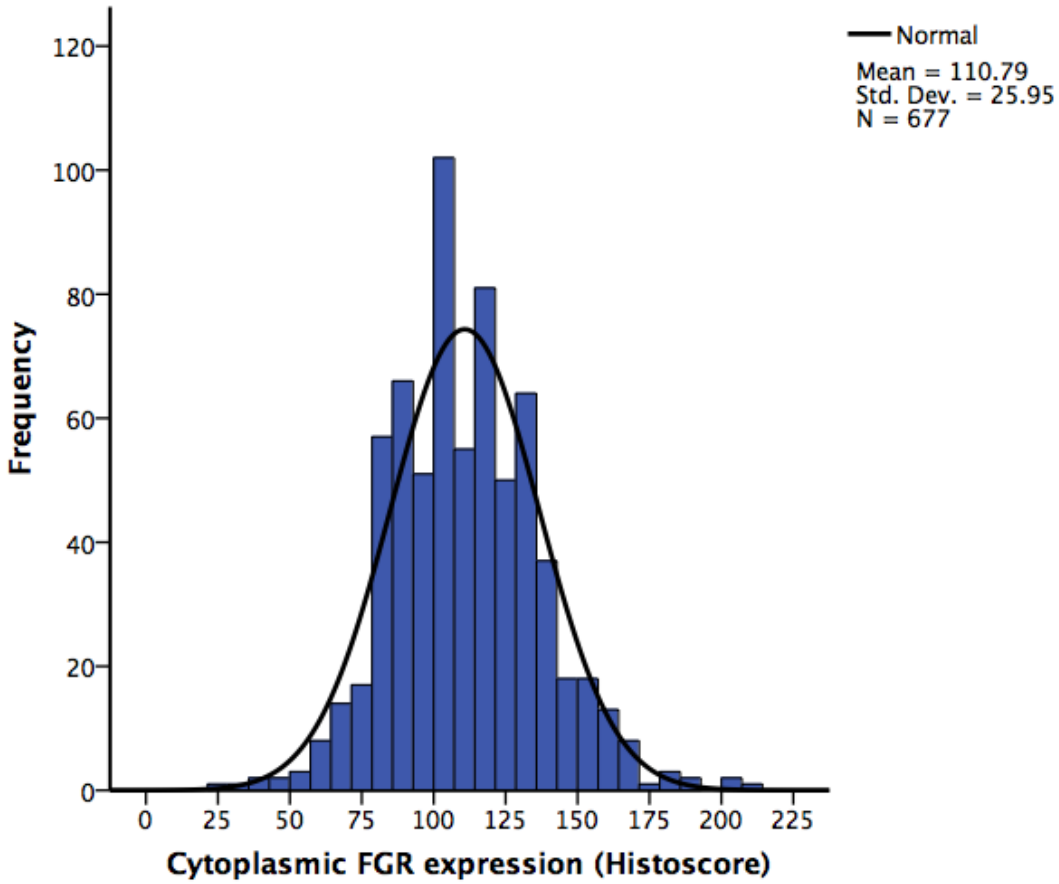
5.4 Validation cohort (cohort 2)

5.4.1 Tumour FGR expression

Tumour FGR was evaluated using immunohistochemistry technology and quantified using the weighted Histoscore method as described previously. None of the tumours demonstrated FGR expression at the cell membrane, however, 100% of tumours had FGR expression in the cytoplasm and nucleus.

5.4.1.1 Cytoplasmic FGR expression

Figure 5.30 demonstrates that cytoplasmic FGR expression does not precisely follow a normal distribution (histogram, figure 5.30), which is supported by a Shapiro-Wilk statistic of 0.990, df 677, p<0.001. The measurements ranged from 26.67 – 183.33 with a median of 110.00 (IQR 73.33 – 146.67). Using the dichotomisation process described in section 5.3.4.1, 21.4% of patients were considered high expressers (above the upper quartile).



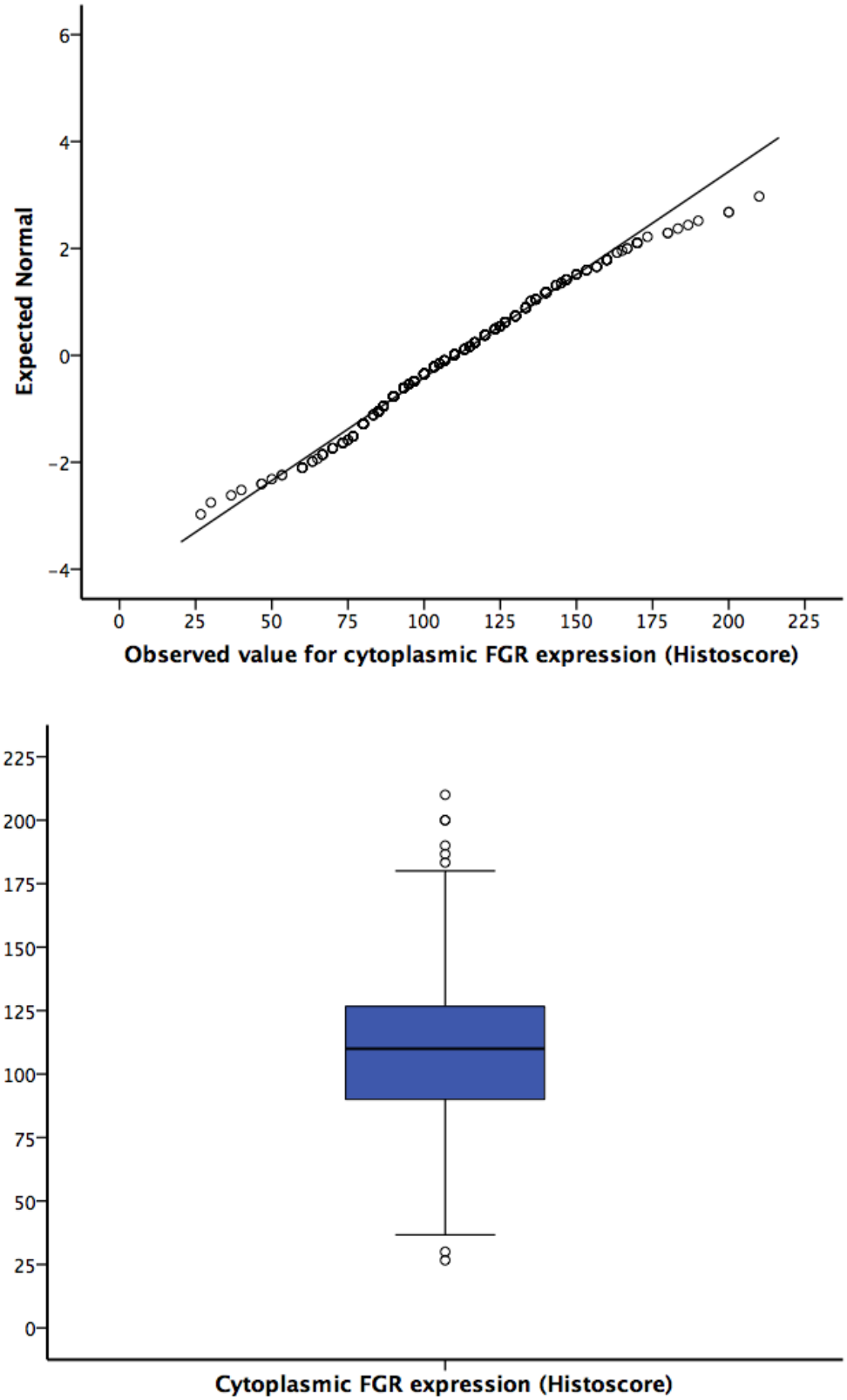


Figure 5.30: Distribution of measurements of cytoplasmic FGR in patients with colorectal cancer

Histogram demonstrating the distribution of cytoplasmic FGR measurements. Q-Q Plot of cytoplasmic FGR measurements. Boxplot demonstrating the median measurement with interquartile range and outliers.

5.4.1.2 Cytoplasmic FGR association with clinicopathological factors

Assessment of cytoplasmic FGR as a continuous variable across different groups of categorical clinicopathological factors

Only advancing T-stage (p=0.025, Kruskal-Wallis) and advancing TNM stage (p=0.043, Kruskal-Wallis) showed a trend towards an association with higher cytoplasmic FGR expression, however, these were not significant following Bonferroni adjustment (p<0.0045). Gender (p=0.376, Mann-Whitney U), presentation (p=0.429, Mann-Whitney U), tumour site (p=0.669, Kruskal-Wallis), N-stage (p=0.567, Kruskal-Wallis), categorical serum CRP (p=0.567, Mann-Whitney U), categorical serum albumin (p=0.824, Mann-Whitney U) and Klintrup score (p=0.283, Kruskal-Wallis) were not associated with continuous cytoplasmic FGR expression.

Assessment of cytoplasmic FGR as a continuous variable across different continuous data types of clinicopathological factors

On Spearman's rank test cytoplasmic FGR was not associated with age (SCC -0.053, p=0.168), serum CRP (SCC -0.009, p=831) or serum albumin (SCC -0.018, p=0.669)

Assessment of cytoplasmic FGR associations with clinicopathological factors: categorical data type

Raised cytoplasmic FGR was not associated with any of the clinicopathological factors studied following Bonferroni adjustment (p<0.0032). Gender (p=0.139), presentation (p=0.103), tumour site (colon vs rectum) (p=0.288), tumour site (right vs left vs rectum) (p=0.597), tumour differentiation (p=0.366), T-stage (p=0.153), N-stage (p=0.579), advancing TNM stage (p=0.020), serum CRP (p=0.452), serum albumin (p=0.886), Klintrup score (p=0.858) and MSI status (p=0.822) were not associated with categorical cytoplasmic FGR expression (table 5.1).

Table 5.1: The relationship between tumour cytoplasmic FGR expression and clinicopathological factors

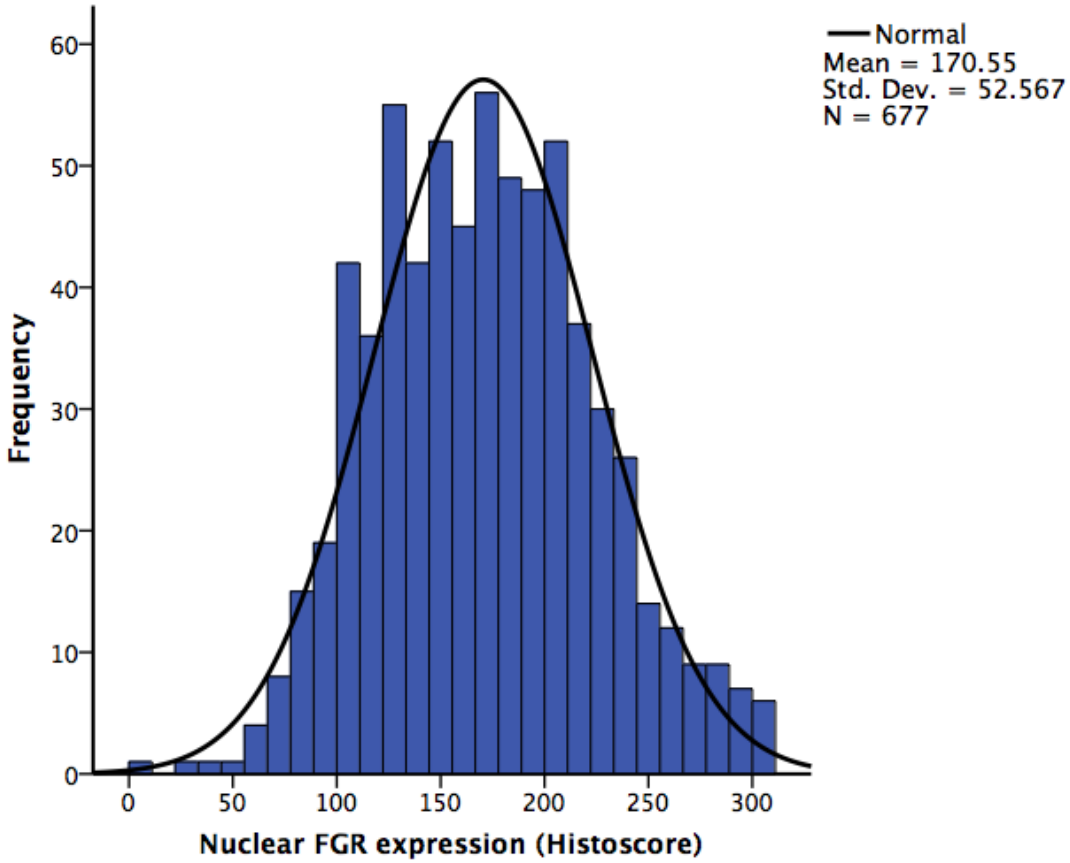
Clinicopathological factors		Low cytoplasmic FGR expression	High cytoplasmic FGR expression	p-value
Sex				
Female		253 (47.6%)	79 (54.5%)	0.139
Male		279 (52.4%)	66 (45.5%)	
Presentation				
Elective		382 (71.8%)	94 (64.8%)	0.103
Emergency		150 (28.2%)	51 (35.2%)	
Tumour Site				
Colon		415 (78.0%)	119 (82.1%)	0.288
Rectum		117 (22.0%)	26 (17.9%)	
Tumour Site				
Right		235 (44.2%)	67 (46.2%)	0.597
Left		181 (34.0%)	52 (35.9%)	
Rectum		116 (21.8%)	26 (17.9%)	
Differentiation				
Well-Mod		483 (90.8%)	128 (88.3%)	0.366
Poor		49 (9.2%)	17 (11.7%)	
T stage				
1		25 (4.7%)	2 (1.4%)	0.153
2		76 (14.3%)	15 (10.3%)	
3		277 (52.1%)	84 (57.9%)	
4		154 (28.9%)	44 (30.3%)	
N stage				
0		343 (64.5%)	96 (66.2%)	0.579
1		129 (24.2%)	37 (25.5%)	
2		60 (11.3%)	12 (8.3%)	
TNM stage (simplified)				
I		92 (17.3%)	13 (9.0%)	0.020
II		245(46.1%)	82 (56.6%)	
III		195 (36.7%)	50 (34.5%)	
Serum CRP				
Normal		203 (49.8%)	67 (53.6%)	0.452
High		205 (50.2%)	58 (46.4%)	
Serum albumin				
Normal		317 (73.2%)	96 (73.8%)	0.886
Low		116 (26.8%)	34 (26.2%)	
Klintrup score				
Good		371 (69.7%)	100 (69.0%)	0.858
Poor		161 (30.3%)	45 (31.0%)	
MSI status				
CI		466 (87.6%)	126 (86.9%)	0.822
MSI		66 (12.4%)	19 (13.1%)	

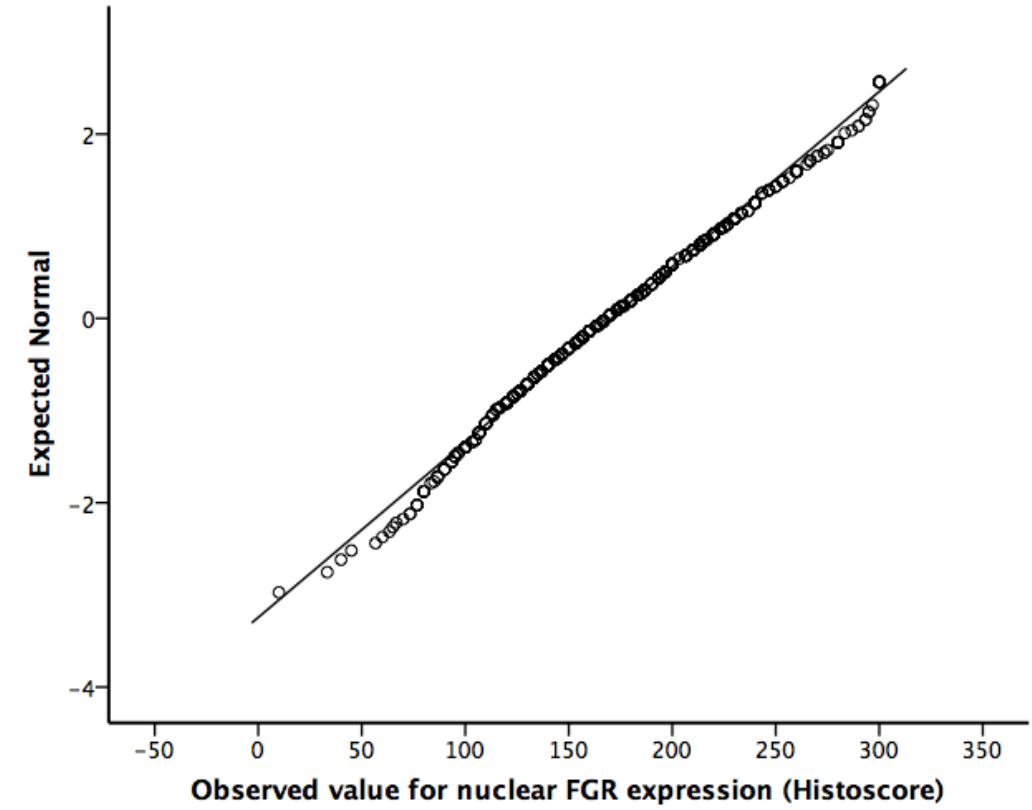
Bonferroni adjustment = <0.0032

* Fishers exact test

5.4.1.3 Nuclear FGR expression

Figure 5.31 demonstrates that the expression of nuclear FGR does not precisely follow a normal distribution (histogram, figure 5.31) which is supported by a Shapiro-Wilk statistic of 0.993, df 677, p=0.003. The measurements ranged from 10.0 – 300.0 with a median of 166.67 (IQR 90.0 – 243.34). Using the dichotomisation process described in section 5.3.4.2, 23.8% of patients were considered high expressers (above the upper quartile).





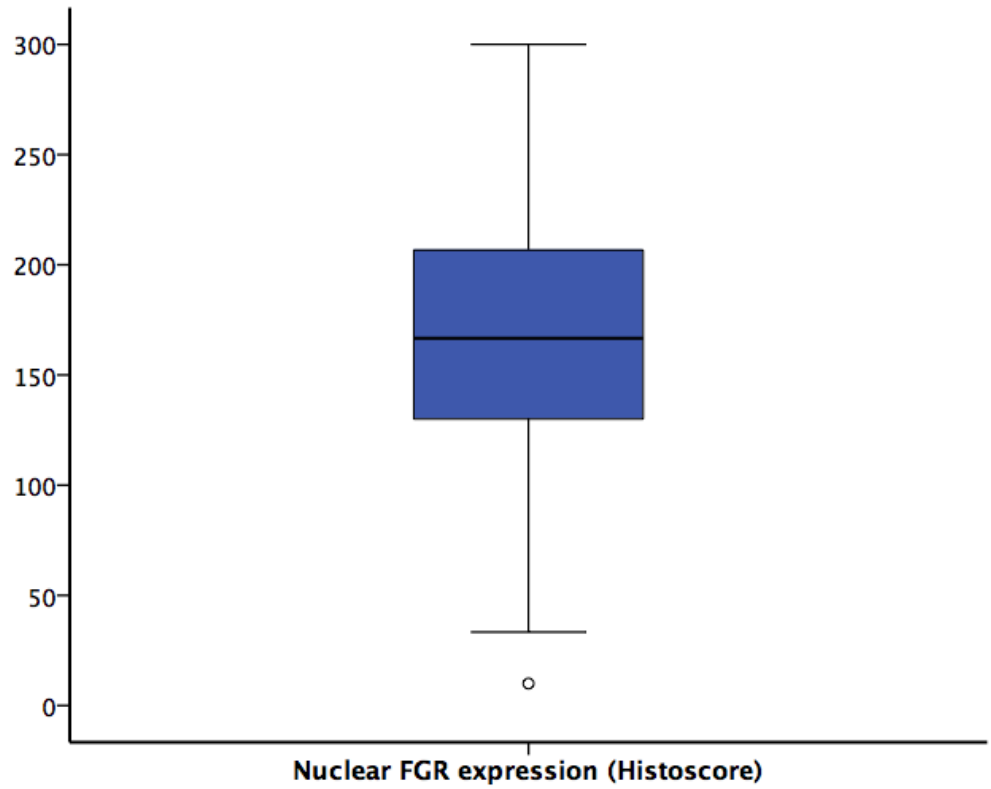


Figure 5.31: Distribution of measurements of nuclear FGR expression in patients with colorectal cancer

Histogram demonstrating the distribution of nuclear FGR measurements. Q-Q Plot of nuclear FGR measurements. Boxplot demonstrating the median measurement with interquartile range and outliers.

5.4.1.4 Nuclear FGR association with clinicopathological factors

Assessment of nuclear FGR as a continuous variable across different groups of categorical clinicopathological factors

Only advancing T-stage (p=0.006, Kruskal-Wallis) and advancing TNM stage (p=0.050, Kruskal-Wallis) showed a trend towards an association with higher nuclear FGR expression, however, these were not significant following Bonferroni adjustment p<0.0042. Gender (p=0.703, Mann-Whitney U), presentation (p=0.066, Mann-Whitney U), tumour site (p=0.834, Kruskal-Wallis), tumour differentiation (p=0.862), N-stage (p=0.779, Kruskal-Wallis), categorical serum CRP (p=0.386, Mann-Whitney U), categorical serum albumin (p=0.205, Mann-Whitney U) and Klintrup score (p=0.205, Kruskal-Wallis) were not associated with continuous nuclear FGR expression.

Assessment of nuclear FGR as a continuous variable across different continuous data types of clinicopathological factors

On Spearman's rank test nuclear FGR was not associated with age (SCC 0.010, p=0.797), serum CRP (SCC -0.093, p=0.032) or serum albumin (SCC 0.029, p=0.488) when the Bonferroni adjustment was p<0.017

Assessment of nuclear FGR associations with clinicopathological factors: categorical data type

Nuclear FGR was not associated with any of the categorical clinicopathological factors studied. Gender (p=0.371), presentation (p=0.053), tumour site (colon vs rectum) (p=0.377), tumour site (right vs left vs rectum) (p=0.879), tumour differentiation (p=0.832), T-stage (p=0.064), N-stage (p=0.842), TNM stage (p=0.163), serum CRP (p=0.748), serum albumin (p=0.133), Klintrup score (p=0.240) and MSI status (p=0.627) were not associated with categorical cytoplasmic FGR expression (table 5.2).

Table 5.2: The relationship between nuclear FGR expression and clinicopathological factors

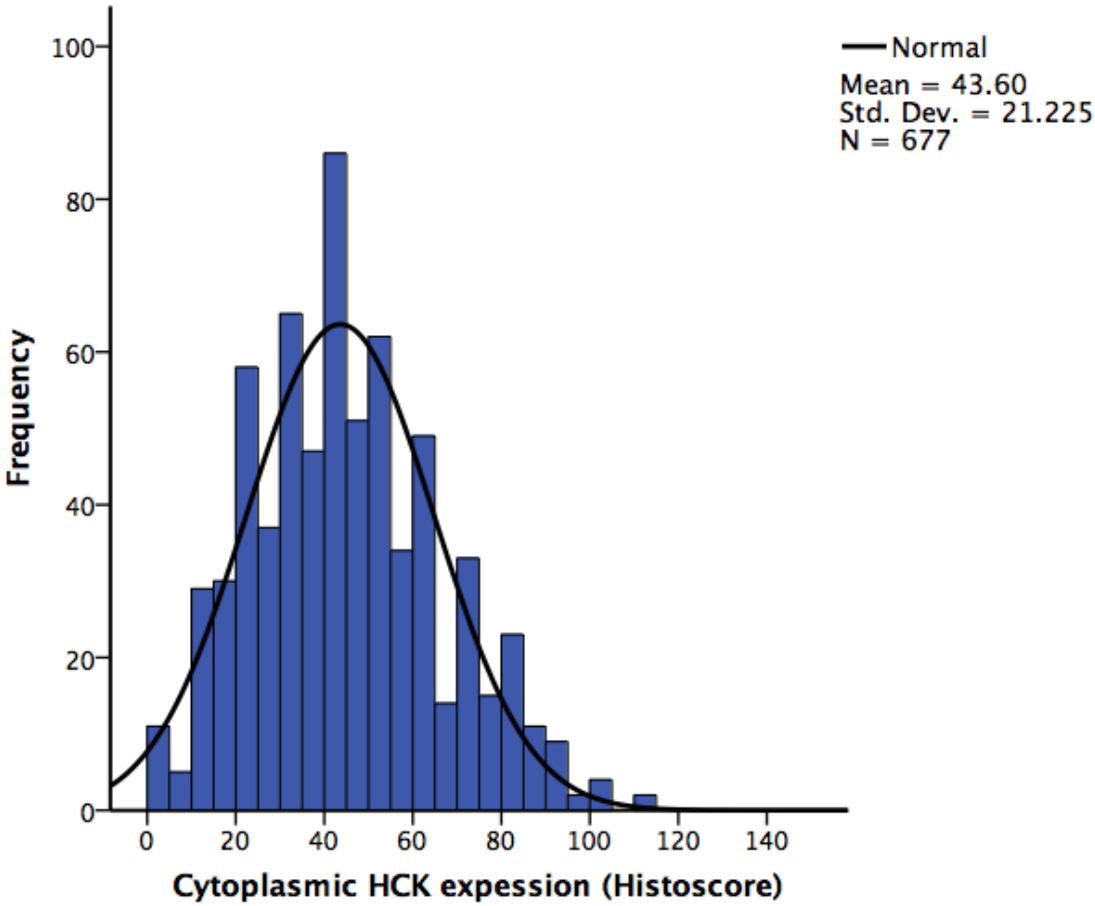
Clinicopathological factors		Low cytoplasmic FRG expression	High cytoplasmic FGR expression	p-value
Sex				
Female		258 (50.0%)	74 (46.0%)	0.371
Male		258 (50.0%)	87 (54.0%)	
Presentation				
Elective		353 (68.4%)	123 (76.4%)	0.053
Emergency		163 (31.6%)	38 (23.6%)	
Tumour Site				
Colon		411 (79.7%)	123 (76.4%)	0.377
Rectum		105 (20.3%)	38 (23.6%)	
Tumour Site				
Right		232 (45.0%)	70 (43.5%)	0.879
Left		178 (34.5%)	55 (34.2%)	
Rectum		106 (20.5%)	36 (22.4%)	
Differentiation				
Well-Mod		465 (90.1%)	146 (90.7%)	0.832
Poor		51 (9.9%)	15 (9.3%)	
T stage				
1		15 (2.9%)	12 (7.5%)	0.064
2		68 (13.2%)	23 (14.3%)	
3		277 (53.7%)	84 (52.2%)	
4		156 (30.2%)	42 (26.1%)	
N stage				
0		335 (64.9%)	104 (64.6%)	0.842
1		128 (24.8%)	38 (23.6%)	
2		53 (10.3%)	19 (11.8%)	
TNM stage (simplified)				
I		73 (14.1%)	32 (19.9%)	0.163
II		257 (49.8%)	70 (43.5%)	
III		186 (36.0%)	59 (36.6%)	
Serum CRP				
Normal		200 (50.3%)	70 (51.9%)	0.748
High		198 (49.7%)	65 (48.1%)	
Serum albumin				
Normal		299 (71.7%)	114 (78.1%)	0.133
Low		118 (28.3%)	32 (21.9%)	
Klintrup score				
Good		353 (68.4%)	118 (73.3%)	0.240
Poor		163 (31.6%)	43 (26.7%)	
MSI status				
CI		453 (87.8%)	139 (86.3%)	0.627
MSI		63 (12.2%)	22 (13.7%)	

Bonferroni adjustment = <0.0042

* Fishers exact test

5.4.2 Tumour HCK expression

Cancer cell HCK expression was evaluated using immunohistochemistry and scored using the weighted histoscore as previously described. HCK was only seen in the cell cytoplasm. Figure 5.32 demonstrates that the expression of cytoplasmic HCK does not precisely follow a normal distribution (histogram, figure 5.32) which is supported by a Shapiro-Wilk statistic of 0.985, df 677, $p<0.001$. The measurements ranged from 0.0 – 110.0 with a median of 40.0 (IQR 10.0 – 30.0 g/l). Using the dichotomisation process described in section 5.3.4.3, 24.2% of patients were considered high expressers (above the upper quartile).



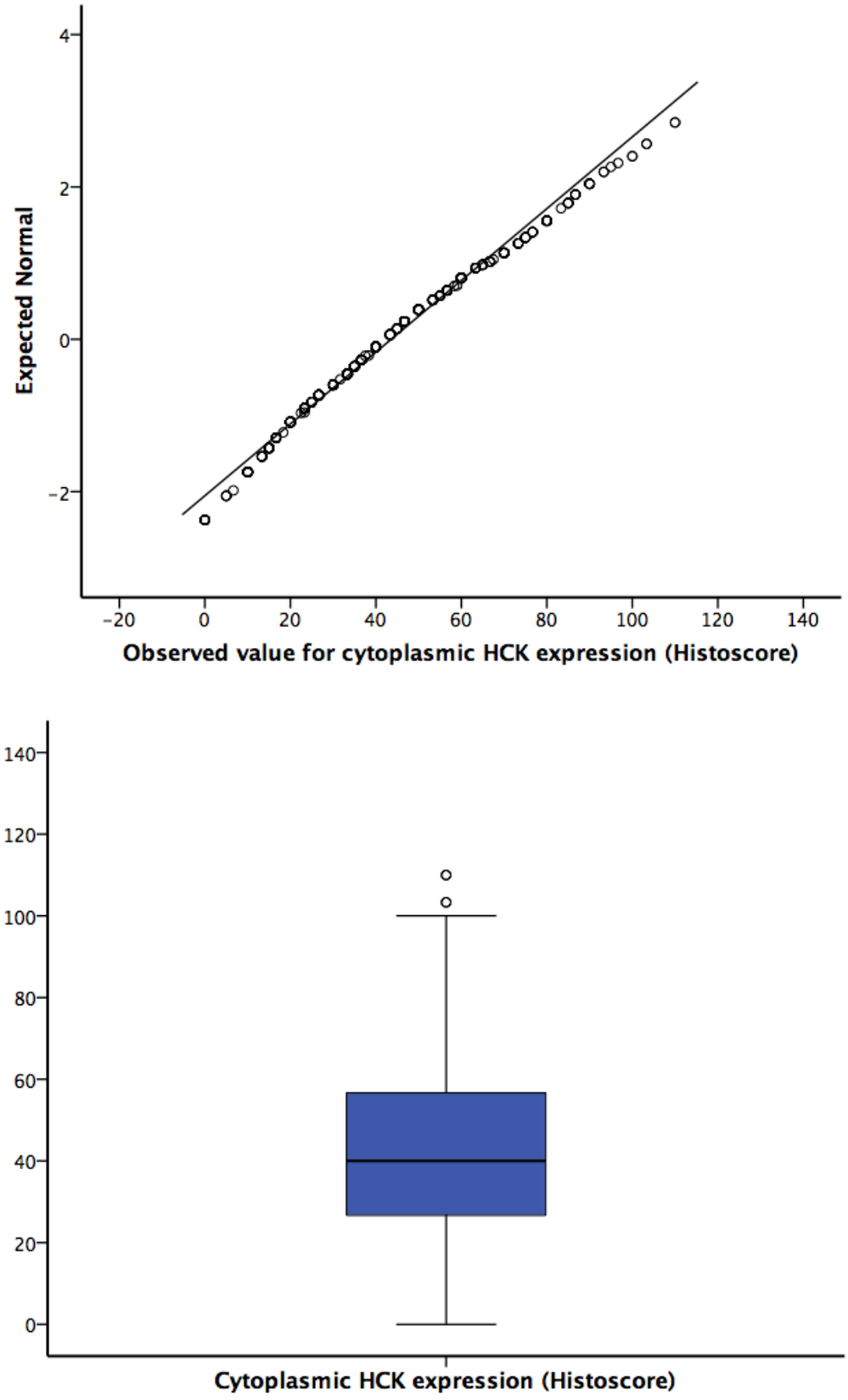


Figure 5.32: Distribution of measurements of cytoplasmic HCK in patients with colorectal cancer

Histogram demonstrating the distribution of cytoplasmic HCK measurements. Q-Q Plot of cytoplasmic HCK measurements. Boxplot demonstrating the median measurement with interquartile range and outliers.

5.4.2.1 Cytoplasmic HCK association with clinicopathological factors

Assessment of cytoplasmic HCK as a continuous variable across different groups of categorical clinicopathological factors

Advancing T-stage (p=0.022, Kruskal-Wallis) showed a trend towards an association with higher cytoplasmic HCK expression, however only advancing TNM stage (p=0.003, Kruskal-Wallis, figure 5.33) was significant following Bonferroni adjustment p<0.0042. Gender (p=0.879, Mann-Whitney U), presentation (p=0.251, Mann-Whitney U), tumour site (p=0.386, Kruskal-Wallis), tumour differentiation (p=0.150), N-stage (p=0.401, Kruskal-Wallis), categorical serum CRP (p=0.367, Mann-Whitney U), categorical serum albumin (p=0.764, Mann-Whitney U) and Klintrup score (p=0.188, Kruskal-Wallis) were not associated with continuous cytoplasmic expression.

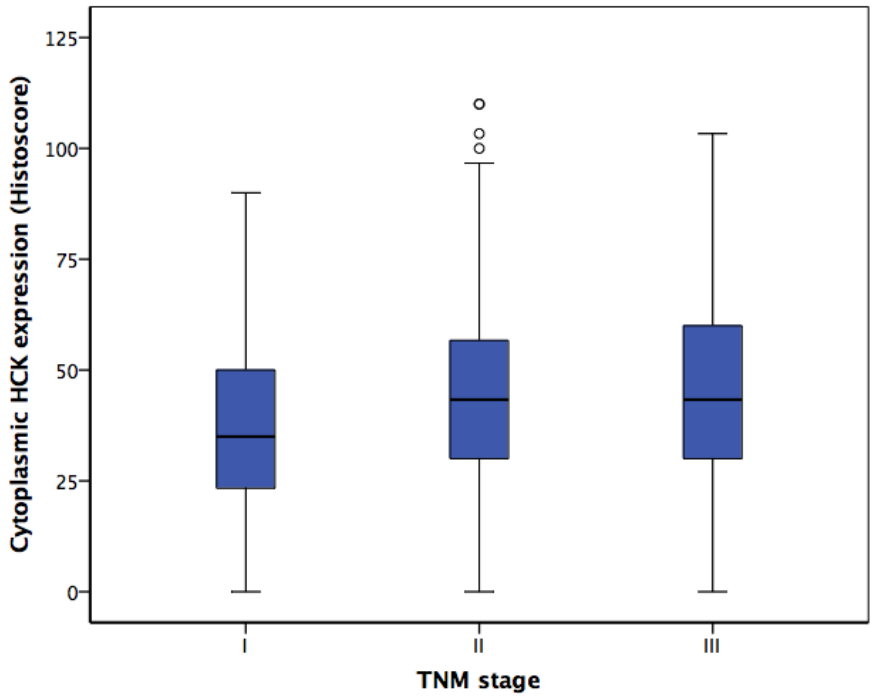


Figure 5.33: The distribution of cytoplasmic HCK expression in TNM stage I, II and III colorectal cancer

Assessment of cytoplasmic HCK as a continuous variable across different continuous data types of clinicopathological factors

On Spearman's rank test cytoplasmic HCK was not associated with age (SCC 0.041, p=0.285), serum CRP (SCC -0.022, p=0.616) or serum albumin (SCC 0.027, p=0.520).

Assessment of cytoplasmic HCK associations with clinicopathological factors: categorical data type

Cytoplasmic HCK was not associated with any of the categorical clinicopathological factors studied. Gender (p=0.798), presentation (p=0.103), tumour site (colon vs rectum) (p=0.215), tumour site (right vs left vs rectum) (p=0.237), tumour differentiation (p=0.130), T-stage (p=0.088), N-stage (p=0.162), TNM stage (p=0.021), serum CRP (p=0.939), serum albumin (p=0.901), Klintrup score (p=0.178) and MSI status (p=0.873) were not associated with categorical cytoplasmic HCK expression (table 5.3).

Table 5.3: The relationship between cytoplasmic HCK expression expression and clinicopathological factors

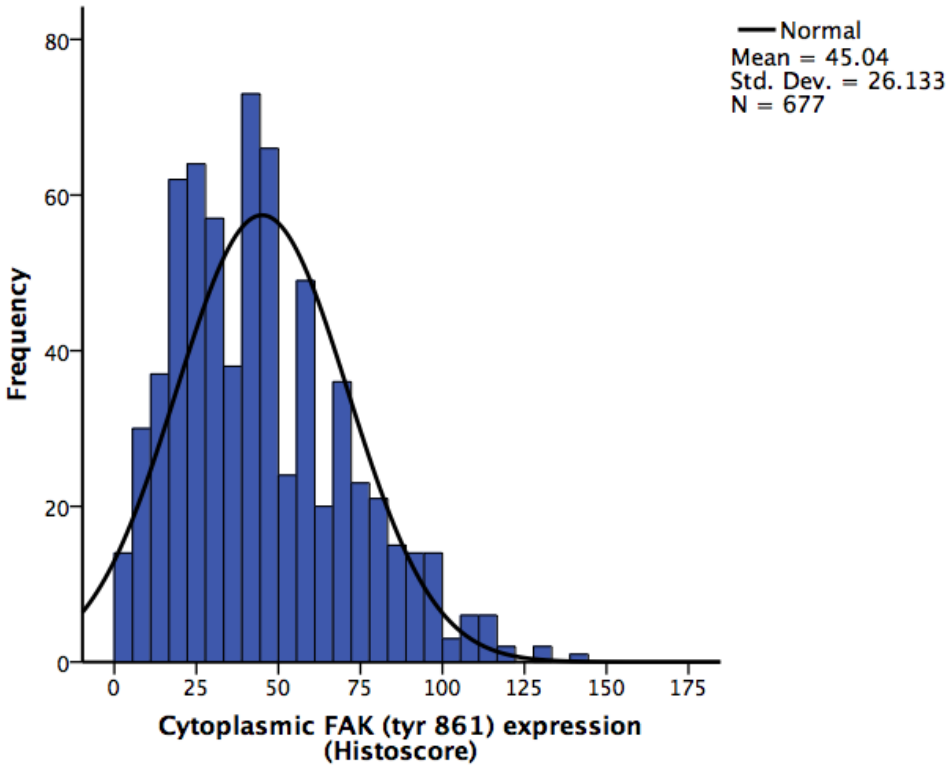
Clinicopathological factors		Low cytoplasmic FRG expression	High cytoplasmic FGR expression	p-value
Sex				
Female		253 (49.3%)	79 (48.2%)	0.798
Male		260 (50.7%)	85 (51.8%)	
Presentation				
Elective		369 (71.9%)	107 (65.2%)	0.103
Emergency		144 (28.1%)	57 (34.8%)	
Tumour Site				
Colon		399 (77.8%)	135 (82.3%)	0.215
Rectum		114 (22.2%)	29 (17.7%)	
Tumour Site				
Right		227 (44.2%)	75 (45.7%)	0.237
Left		171 (33.3%)	62 (37.8%)	
Rectum		115 (22.4%)	27 (16.5%)	
Differentiation				
Well-Mod		468 (91.2%)	143 (87.2%)	0.130
Poor		45 (8.8%)	21 (12.8%)	
T stage				
1		22 (4.3%)	5 (3.0%)	0.088
2		78 (15.2%)	13 (7.9%)	
3		268 (52.2%)	93 (56.7%)	
4		145 (28.3%)	53 (32.3%)	
N stage				
0		342 (66.7%)	97 (59.1%)	0.162
1		117 (22.8%)	49 (29.9%)	
2		54 (10.5%)	18 (11.0%)	
TNM stage (simplified)				
I		90 (17.5%)	15 (9.1%)	0.021
II		247 (48.1%)	80 (48.8%)	
III		176 (34.3%)	69 (42.1%)	
Serum CRP				
Normal		202 (50.8%)	68 (50.4%)	0.939
High		196 (49.2%)	67 (49.6%)	
Serum albumin				
Normal		309 (73.2%)	104 (73.8%)	0.901
Low		113 (26.8%)	37 (26.2%)	
Klintrup score				
Good		350 (68.2%)	121 (73.8%)	0.178
Poor		163 (31.8%)	43 (26.2%)	
MSI status				
CI		448 (87.3%)	144 (87.8%)	0.873
MSI		65 (12.7%)	20 (12.2%)	

Bonferroni adjustment = <0.0042

* Fishers exact test

5.4.3 Tumour FAK (tyr 861) expression

Cancer cell FAK (tyr 861) expression was evaluated using immunohistochemistry and scored using the weighted histoscore as previously described. FAK (tyr 861) was only seen in the cell cytoplasm. Figure 5.34 demonstrates that the expression of cytoplasmic FAK (tyr 861) does not precisely follow a normal distribution (histogram, figure 5.34), which is supported by a Shapiro-Wilk statistic of 0.960, df 677, $p<0.001$. The measurements ranged from 0.0 – 140.0 with a median of 40.0 (IQR 5.0 – 75.0). Using the dichotomisation process described in section 5.3.4.3, 31.3% of patients were considered high expressers (above the upper tertile).



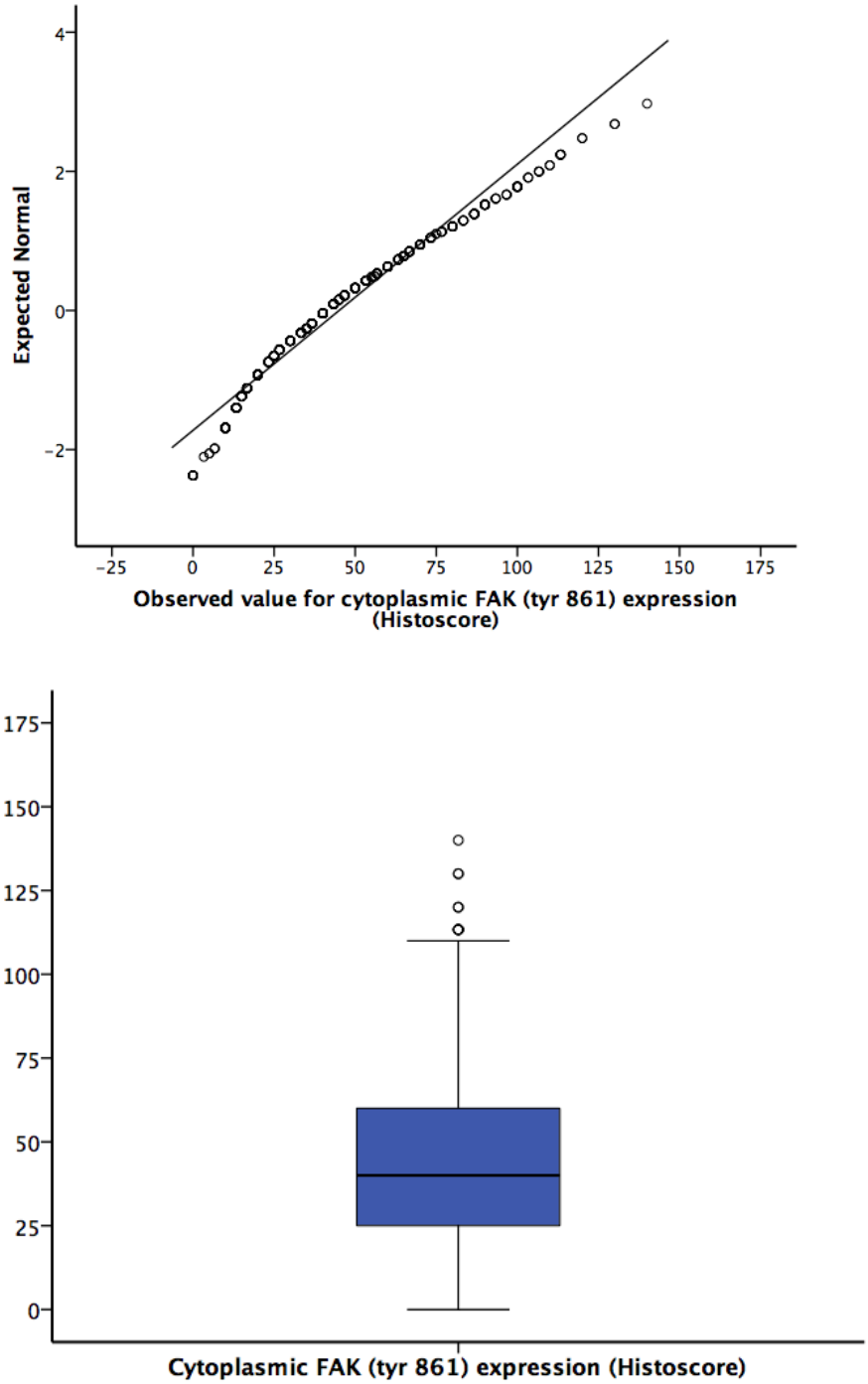


Figure 5.34: Distribution of measurements of cytoplasmic HCK in patients with colorectal cancer

Histogram demonstrating the distribution of cytoplasmic HCK measurements. Q-Q Plot of cytoplasmic HCK measurements. Boxplot demonstrating the median measurement with interquartile range and outliers.

5.4.3.1 Cytoplasmic FAK (tyr 861) association with clinicopathological factors

Assessment of cytoplasmic FAK (tyr 861) as a continuous variable across different groups of categorical clinicopathological factors

Only hypoalbuminaemia was associated with higher cytoplasmic FAK (tyr 861) expression (p=0.001, Bonferroni p<0.0042, figure 5.35). The median cytoplasmic FAK (tyr 861) measurement was 50.00 (IQR 10.00 – 90.00) in the hypoalbuminaemia group compared to 40.00 (IQR 3.33 – 76.67) in patients with a normal serum albumin. Gender (p=0.765, Mann-Whitney U), presentation (p=0.272, Mann-Whitney U), tumour site (p=0.099, Kruskal-Wallis), tumour differentiation (p=0.260), T-stage (p=0.662, Kruskal-Wallis) N-stage (p=0.705, Kruskal-Wallis), TNM stage (p=0.623, Kruskal-Wallis), categorical serum CRP (p=0.929, Mann-Whitney U), Klintrup score (p=0.743, Kruskal-Wallis) were not associated with continuous cytoplasmic FAK (tyr 861) expression.

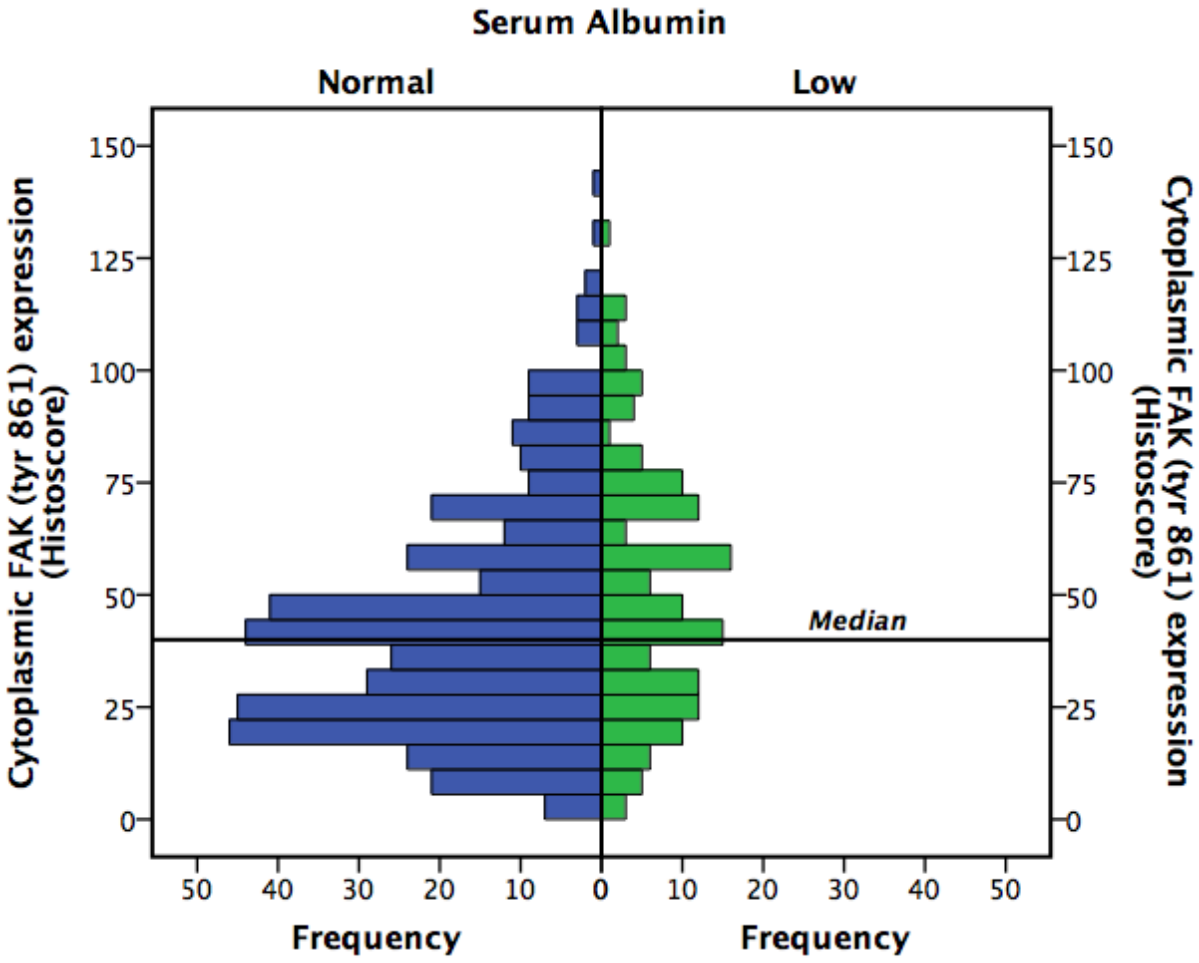


Figure 5.35: Distribution of cytoplasmic FAK (tyr 861) expression stratified by serum albumin measurements.

Assessment of cytoplasmic FAK (tyr 861) as a continuous variable across different continuous data types of clinicopathological factors

On Spearman's rank test cytoplasmic FAK (tyr 861) was not associated with age (SCC -0.021, p=0.592) or serum CRP (SCC -0.023, p=0.590). There was an association with serum albumin (SCC -0.182, p<0.001, figure 5.36) even when the Bonferroni adjustment of p<0.017 was enforced, however, the coefficient is below the a priori SCC of 0.300.

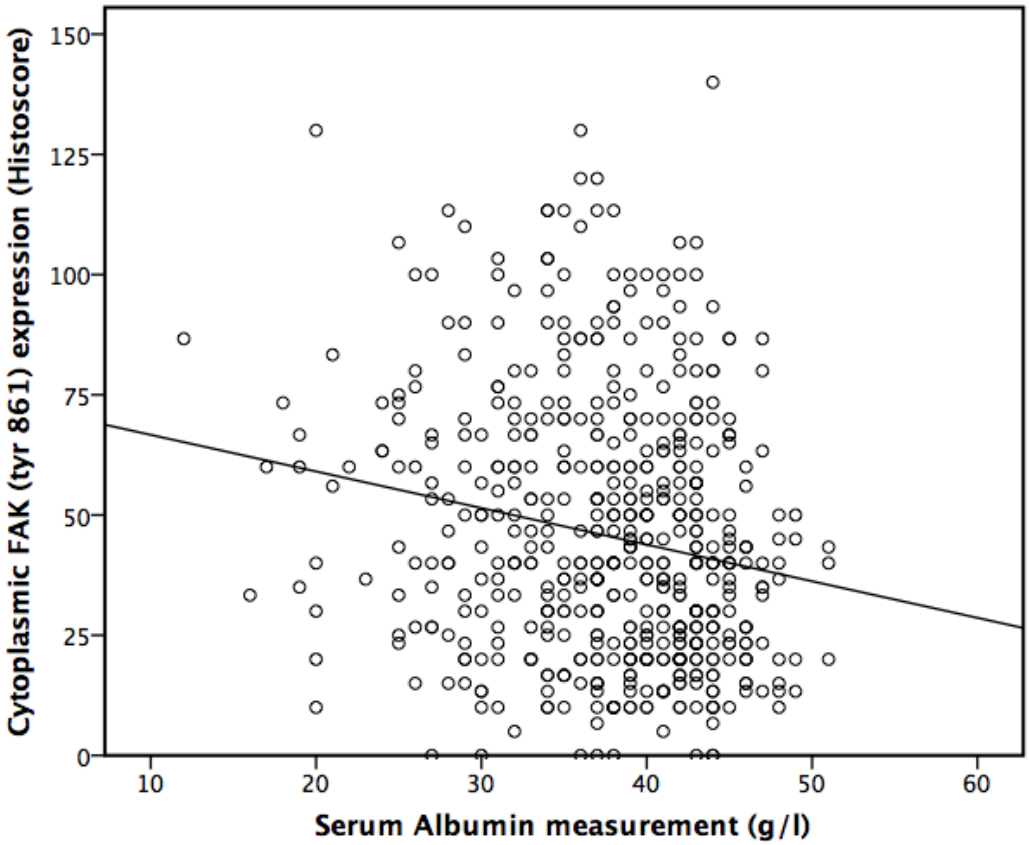


Figure 5.36: Scatter plot of cytoplasmic FAK (tyr 861) expression and serum albumin in patients with colorectal cancer

Assessment of cytoplasmic FAK (tyr 861) associations with clinicopathological factors: categorical data type

Cytoplasmic FAK (tyr 861) was only associated with hypoalbuminaemia (p<0.001). Gender (p=0.873), presentation (p=0.208), tumour site (colon vs rectum) (p=0.443), tumour site (right vs left vs rectum) (p=0.074), tumour differentiation (p=0.077), T-stage (p=0.835), N-stage (p=0.778), TNM stage (p=0.613), serum CRP (p=0.983), Klintrup score (p=0.788) and MSI status (p=0.877) were not associated with categorical cytoplasmic FAK (tyr 861).

Table 5.4: The relationship between cytoplasmic FAK (tyr 861) expression and clinicopathological factors

Clinicopathological factors		Low cytoplasmic FRG expression	High cytoplasmic FGR expression	p-value
Sex				
Female		229 (49.2%)	103 (48.6%)	0.873
Male		236 (50.8%)	109 (51.4%)	
Presentation				
Elective		320 (68.8%)	156 (73.6%)	0.208
Emergency		145 (31.2%)	56 (26.4%)	
Tumour Site				
Colon		363 (78.1%)	171 (80.7%)	0.443
Rectum		102 (21.9%)	41 (19.3%)	
Tumour Site				
Right		217 (46.7%)	85 (40.1%)	0.074
Left		147 (31.6%)	86 (40.6%)	
Rectum		101 (21.7%)	41 (19.3%)	
Differentiation				
Well-Mod		426 (91.6%)	185 (87.3%)	0.077
Poor		39 (8.4%)	27 (12.7%)	
T stage				
1		19 (4.1%)	8 (3.8%)	0.835
2		66 (14.2%)	25 (11.8%)	
3		244 (52.5%)	117 (55.2%)	
4		136 (29.2%)	62 (29.2%)	
N stage				
0		302 (64.9%)	137 (64.6%)	0.778
1		116 (24.9%)	50 (23.6%)	
2		47 (10.1%)	25 (11.8%)	
TNM stage (simplified)				
I		76 (16.3%)	29 (13.7%)	0.613
II		220 (47.3%)	107 (50.5%)	
III		169 (36.3%)	76 (35.8%)	
Serum CRP				
Normal		184 (50.7%)	86 (50.6%)	0.983
High		179 (49.3%)	84 (49.4%)	
Serum albumin				
Normal		298 (77.8%)	115 (63.9%)	<0.001
Low		85 (22.2%)	65 (36.1%)	
Klintrup score				
Good		325 (69.9%)	146 (68.9%)	0.788
Poor		140 (30.1%)	66 (31.1%)	
MSI status				
CI		406 (87.3%)	186 (87.7%)	0.877
MSI		59 (12.7%)	26 (12.3%)	

Bonferroni adjustment = <0.0042

5.4.4 SFK interrelationships

To adjust for multiple correlations we used the Bonferroni method for adjusting alpha for multiple comparisons. For correlations to be significant a p-value must be less than p<0.0083 and a Spearman's correlation coefficient greater than 0.300 or less than -0.300.

Of all the correlations assessed, only higher cytoplasmic HCK expression correlated with higher expression levels of cytoplasmic FAK (tyr 861) (SCC 0.413, p<0.001) (table 5.5, figure 5.37). In addition, higher expression levels of cytoplasmic HCK weakly correlated with higher expression levels of cytoplasmic FGR (SCC 0.289, p<0.001) (table 5.5).

Table 5.5: Spearman correlation coefficients for the assessment of interrelationships between SFK expression and FAK (tyr 861) expression

		nFGR	**cHCK**	**cFAK (tyr861)**
cFGR	SCC P-value	-0.094 0.015	0.289 <0.001	0.142 <0.001
nFGR	SCC P-value		0.037 0.336	0.096 0.012
cHCK	SCC P-value			**0.413** **<0.001**
cFAK861	SCC P-value			

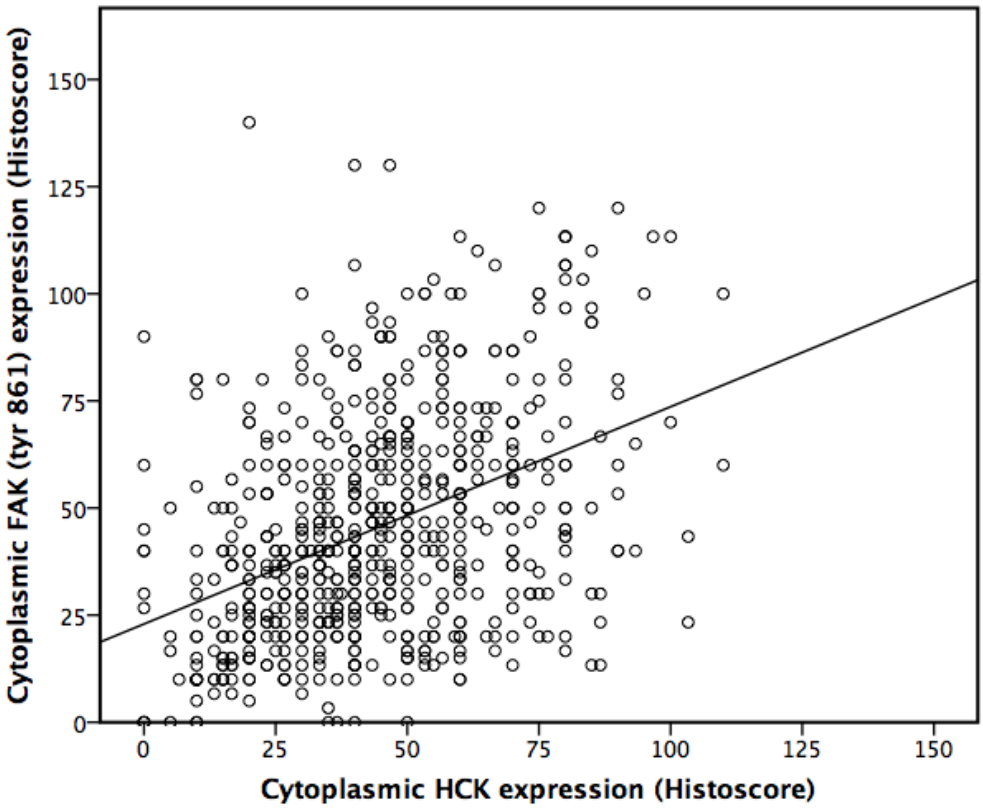


Figure 5.37: Correlation between Cytoplasmic HCK and cytoplasmic FAK (tyr 861) expressions (p<0.001, PCC 0.413)

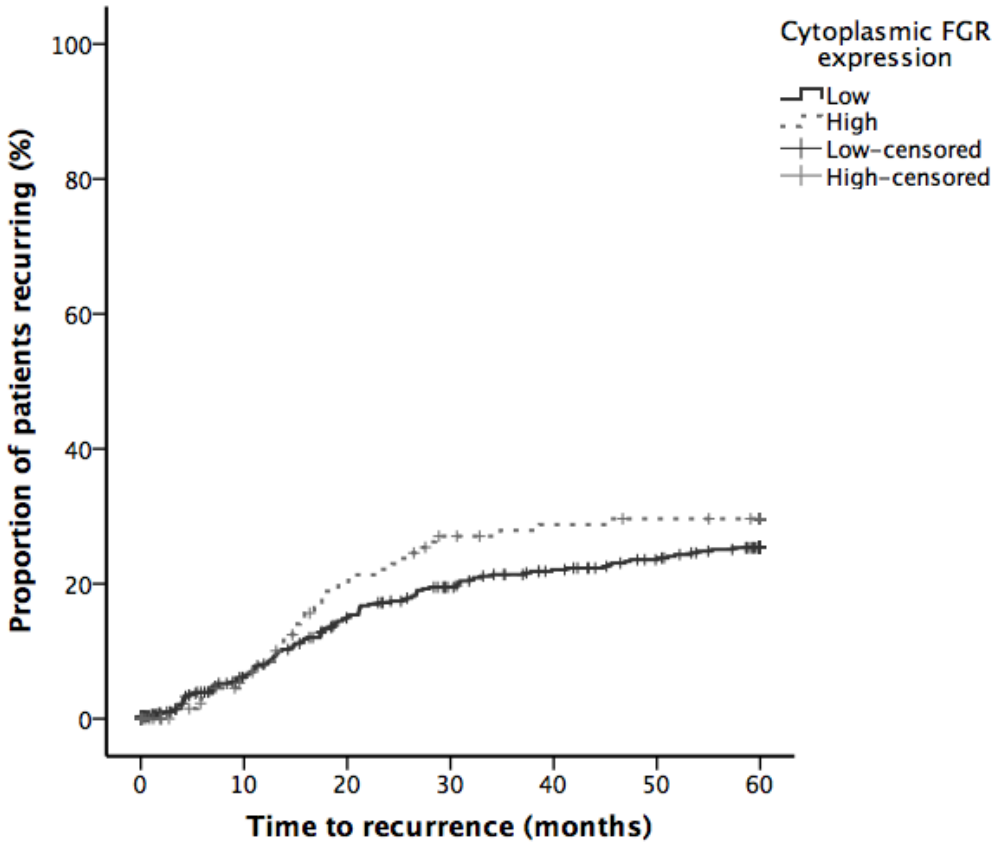
5.4.5 Association of SFK expression, clinicopathological factors and survival

During follow-up there were 150 (22.2%) recurrences and 260 (38.4%) deaths. Five year recurrence-free and overall survival rates for the clinicopathological factors studied can be found in appendix 3.2.

5.4.5.1 Univariable recurrence-free survival - cytoplasmic FGR expression

Tumour cytoplasmic FGR measurements were not significantly different in patients who went on to develop disease recurrence (p=0.817, Mann-Whitney U). The median for patients with recurrence was 110.00 (IQR 69.17 – 150.83) compared with 110.00 (IQR 76.66 – 143.34) in the non-recurrence group (supplementary figure 5.1, appendix 5.3).

The 5 year recurrence-free survival rate for patients with a raised cytoplasmic FGR was 54.5% compared to 53.0% in patients with a normal serum CRP (p=0.752, Pearson's chi square). On logrank analysis, raised cytoplasmic FGR was not significantly associated with poor recurrence-free survival (p=0.301, figure 5.38). The mean survival for patients with a raised cytoplasmic FGR was 47.5 months (95% CI 44.0 – 51.0) compared with 50.0 months (95% CI 48.2 – 51.7) in the low cytoplasmic FGR group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	532	429	377	345	322	305	283
High	145	119	99	87	83	81	79

Figure 5.38: The relationship between cytoplasmic FGR expression and recurrence-free survival in patients with colorectal cancer

Kaplan-Meier curve demonstrating the proportion of patients recurring stratified by cytoplasmic FGR *expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.*

On Cox univariable regression analysis raised cytoplasmic FGR was not significantly associated with poor recurrence-free survival when dichotomised as a categorical variable (HR 1.22 (95% CI 0.84 – 1.76), p=0.302). The hazard ratio remained non-significant when it was included as a continuous variable (HR 1.00 (95% CI 0.99 – 1.01), p=0.761). In addition to the non-significant associations observed between cytoplasmic FGR expression and disease recurrence, its predictive value remains poor. When ROC analysis was performed using recurrence as the endpoint, the AUC was 0.49 (95% CI 0.44 – 0.55, p=0.817) for cytoplasmic FGR as a continuous variable and an AUC of 0.52 (95% CI 0.47 – 0.57, p=0.435) for cytoplasmic FGR as a categorical variable (supplementary figure 5.2, appendix 5.3).

5.4.5.2 Univariable recurrence-free survival - Nuclear FGR expression

There was no statistically significant difference between nuclear FGR measurements of patients who did and did not develop disease recurrence during the follow up period (p=0.777, Mann-Whitney U). The median nuclear FGR measurement was 166.67 (IQR 89.17 – 244.17) in the recurrence group compared to 170.00 (IQR 93.33 – 246.67) in the non-recurrence group (supplementary figure 5.3, appendix 5.3).

The 5 year recurrence-free survival rate for patients with a raised nuclear FGR was 55.3% compared to 52.7% in patients with a low nuclear FGR expression (p=0.569, Pearson's chi square). On logrank analysis raised nuclear FGR was not associated with poor recurrence-free survival (p=0.878). The mean survival for patients with raised nuclear FGR was 48.8 months (95% CI 45.5 – 52.0) compared with 49.6 months (95% CI 47.9 – 51.4) in the low nuclear FGR group (supplementary figure 5.4, appendix 5.3).

On Cox univariable regression analysis nuclear FGR was not associated with poor recurrence free survival when dichotomised as a categorical variable (HR 1.03 (95% CI 0.71 – 1.49), p=0.878). Furthermore, the hazard ratio remained non-significant when it was included as a continuous variable (HR 1.00 (95% CI 1.00 – 1.00), p=0.621). In addition to the non-significant associations observed between nuclear FGR and disease recurrence, its predictive value is relatively poor. When ROC analysis was performed using recurrence as the endpoint, the AUC was 0.51 (95% CI 0.46 – 0.56, p=0.777) for nuclear FGR as a continuous variable and an AUC of 0.51 (95% CI 0.45 – 0.56, p=0.832) for nuclear FGR as a categorical variable (supplementary figure 5.5, appendix 5.3).

5.4.5.3 Univariable recurrence-free survival - cytoplasmic HCK expression

Tumour cytoplasmic HCK measurements were higher in patients who went on to develop disease recurrence (p=0.021, Mann-Whitney U, figure 5.39). The median for patients with recurrence was 45.00 (IQR 14.17 – 75.83) compared with 40.00 (IQR 10.00 – 50.00) in the non-recurrence group.

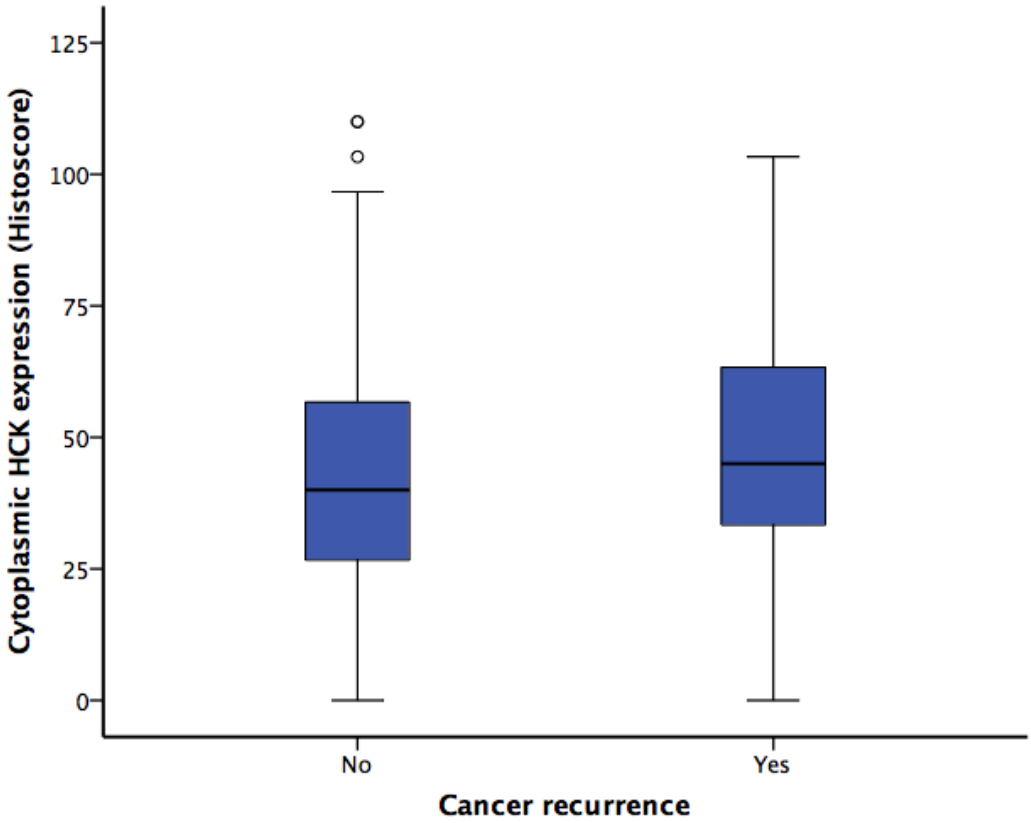
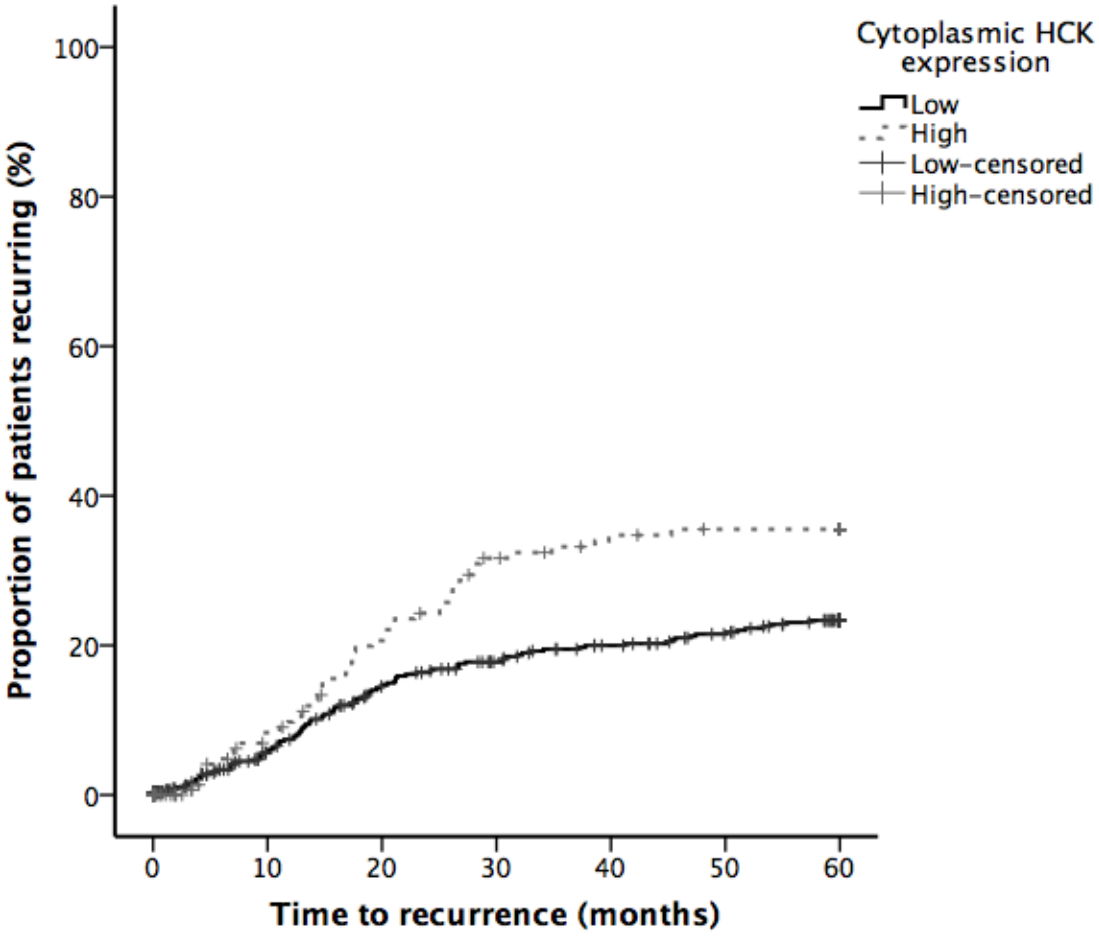


Figure 5.39: The distribution of cytoplasmic HCK measurements in patients with and without disease recurrence (p=0.021)

The 5 year recurrence-free survival rate for patients with a raised cytoplasmic HCK was 49.4% compared to 54.6% in patients with a low cytoplasmic HCK expression (p=0.246, Pearson's chi square). On logrank analysis, raised cytoplasmic HCK was significantly associated with poor recurrence free survival (p=0.004, figure 5.40). The mean survival for patients with a raised cytoplasmic HCK was 45.2 months (95% CI 41.8 – 48.7) compared with 50.7 months (95% CI 49.0 – 52.4) in the low cytoplasmic HCK group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	513	419	367	341	321	305	280
High	164	129	109	91	84	81	81

Figure 5.40: The relationship between cytoplasmic HCK expression and recurrence-free survival in patients with colorectal cancer

Kaplan-Meier curve demonstrating the proportion of patients recurring stratified by cytoplasmic HCK expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis raised cytoplasmic HCK was significantly associated with poor recurrence free survival when dichotomised as a categorical variable (HR 1.64 (95% CI 1.17 – 2.31), p=0.004). The significance of the hazard ration remained when it was included as a continuous variable (HR 1.01 (95% CI 1.00 – 1.02), p=0.005). Despite the significant associations observed between cytoplasmic HCK and disease recurrence, its predictive value remains poor. When ROC analysis was performed using recurrence as the endpoint, the AUC was 0.56 (95% CI 0.51 – 0.61, p=0.022) for cytoplasmic HCK as a continuous variable and an AUC of 0.55 (95% CI 0.50 – 0.61, p=0.043) for cytoplasmic HCK as a categorical variable (figure 5.41).

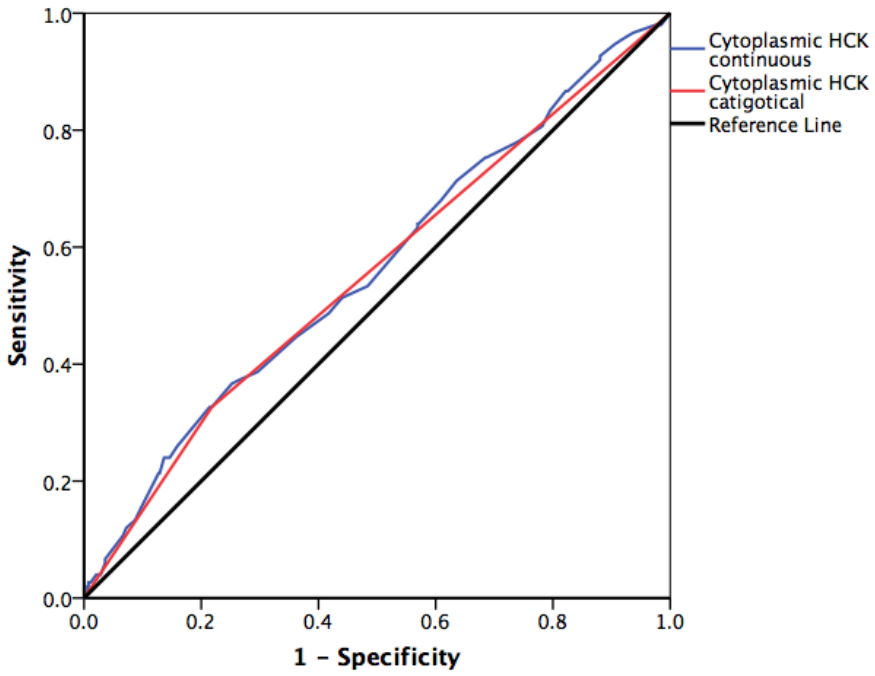


Figure 5.41: The predictive value of cytoplasmic HCK in identifying patients who will develop cancer recurrence

Receiver-operator-characteristic curve demonstrating the predictive value of cytoplasmic HCK in identifying patients who will develop disease recurrence.

5.4.5.4 Univariable overall survival - cytoplasmic FGR expression

Cytoplasmic FGR measurements were not statistically higher in patients who died during the follow up period (p=0.783, Mann-Whitney U). The median for patients who died was 110.00 (IQR 70.00 – 150.00) compared with 110.00 (IQR 74.99 – 140.01) in the alive group (supplementary figure 5.6, appendix 5.3).

The 5 year overall survival rate for patients with a raised cytoplasmic FGR expression was 58.6% compared to 57.1% in patients with a low cytoplasmic FGR expression (p=0.750, Pearson's chi square). On logrank analysis, raised cytoplasmic FGR expression was not significantly associated with poor overall survival (p=0.683). The mean survival for patients with a raised cytoplasmic FGR expression was 44.6 months (95% CI 41.0 – 48.2) compared with 46.2 months (95% CI 44.3 – 48.0) in the low cytoplasmic FGR expression group (supplementary figure 5.7, appendix 5.3).

On Cox univariable regression analysis raised cytoplasmic FGR was not significantly associated with poor overall survival when dichotomised as a categorical variable (HR 1.06 (95% CI 0.79 – 1.42), p=0.683) or when analysed as a continuous variable (HR 1.00 95% CI 1.00 – 1.01, p=0.832). In addition to the non-significant

associations with survival, cytoplasmic FGR did not predict death during the follow up period as a categorical variable (AUC was 0.51 (95% CI 0.46 – 0.55, p=0.783), ROC analysis) or continuous variable (AUC was 0.51 (95% CI 0.46 – 0.55, p=0.752), ROC analysis), (supplementary figure 5.8, appendix 5.3).

5.4.5.5 Univariable overall survival - nuclear FGR expression

Nuclear FGR measurements were not significantly different in patients who died during the follow up period (p=0.633, Mann-Whitney U). The median nuclear FGR was 166.67 (IQR 90 - 243.34) in the alive group compared to 170.00 (IQR 90.83 – 249.17) in patients who died during follow-up (supplementary figure 5.9, appendix 5.3).

The 5 year overall survival rate for patients with a low nuclear FGR expression was 55.9% compared to 57.9% in patients with a low nuclear FGR expression (p=0.647, Pearson's chi square). On logrank analysis nuclear FGR was not associated with poor overall survival (p=0.560). The mean survival for patients with raised nuclear FGR was 45.5 months (95% CI 42.2 – 48.8) compared with 46.0 months (95% CI 44.1 – 47.8) in the low nuclear FGR group (supplementary figure 5.10, appendix 5.3).

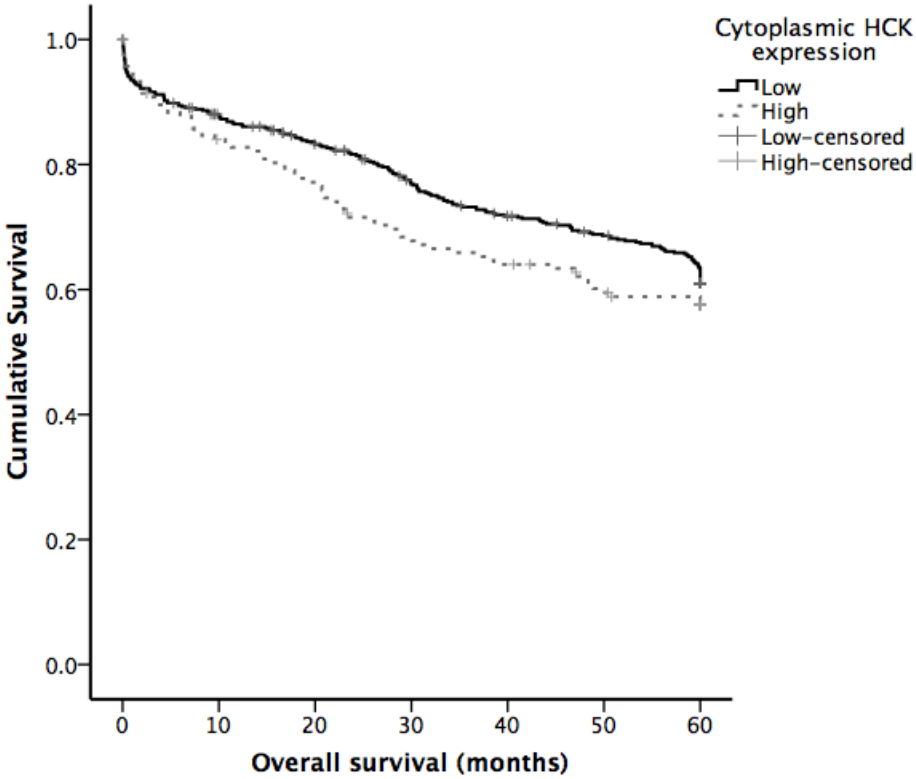
On Cox univariable regression analysis nuclear FGR was not associated with poor overall survival when dichotomised as a categorical variable (HR 1.09 (95% CI 0.82 – 1.44), p=0.560) or when included as a continuous variable (HR 1.00 (95% CI 1.00 – 1.00), p=0.609). Furthermore, nuclear FGR did not offer any predictive value when death was used as an endpoint. the AUC was 0.51 (95% CI 0.47 – 0.56, p=0.634) for nuclear FGR as a continuous variable and an the AUC was 0.51 (95% CI 0.47 – 0.56, p=0.569) for nuclear FGR as a categorical variable (supplementary figure 5.11, appendix 5.3).

5.4.5.6 Univariable overall survival - cytoplasmic HCK expression

Cytoplasmic HCK measurements were not higher in patients who died during the follow up period (p=0.240, Mann-Whitney U). The median for patients who died was 43.33 (IQR 13.33 – 73.33) compared with 40.00 (IQR 10.00– 70.00 mg/l) in the alive group (supplementary figure 5.12, appendix 5.3.).

The 5 year overall survival rate for patients with a raised cytoplasmic HCK expression was 54.3% compared to 58.5% in patients with a low cytoplasmic HCK expression (p=0.342, Pearson's chi square). On logrank analysis, raised cytoplasmic HCK expression was not associated with poor overall survival (p=0.243, figure 5.42). The mean survival for patients with a raised cytoplasmic HCK was 42.9 months (95% CI 39.4 – 46.5)

compared with 46.8 months (95% CI 44.9 – 48.6) in the low cytoplasmic HCK group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	513	442	411	373	345	327	301
High	164	134	124	109	102	92	89

Figure 5.42: The relationship between cytoplasmic HCK expression and overall survival in patients with colorectal cancer

Kaplan-Meier curve demonstrating the proportion of patients surviving stratified by cytoplasmic HCK expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis raised cytoplasmic HCK expression was not associated with poor overall survival when dichotomised as a categorical variable (HR 1.18 (95% CI 0.89 – 1.55), p=0.245) or as a continuous variable (HR 1.01 95% CI 1.00 – 1.01, p=0.101). Furthermore, cytoplasmic HCK did not offer any predictive value for death when studied as a continuous variable (AUC was 0.53 (95% CI 0.48 – 0.57, p=0.240)) or a categorical variable (AUC of 0.52 (95% CI 0.47 – 0.56, p=0.493)) (supplementary figure 5.13, appendix 5.3).

5.4.6 The relationship between SRC activation and survival

5.4.6.1 Univariable recurrence-free survival - cytoplasmic FAK (tyr 861) expression

There was no statistically significant difference between the cytoplasmic FAK (tyr 861) expression of patients who did and did not develop disease recurrence during follow-up (p=0.051, Mann-Whitney U, figure 5.43). The median cytoplasmic FAK (tyr 861) expression was 40.00 (IQR 4.33 – 76.67) in the non-recurrence group compared to 40.00 (IQR 0.00 – 80.83 in the recurrence group.

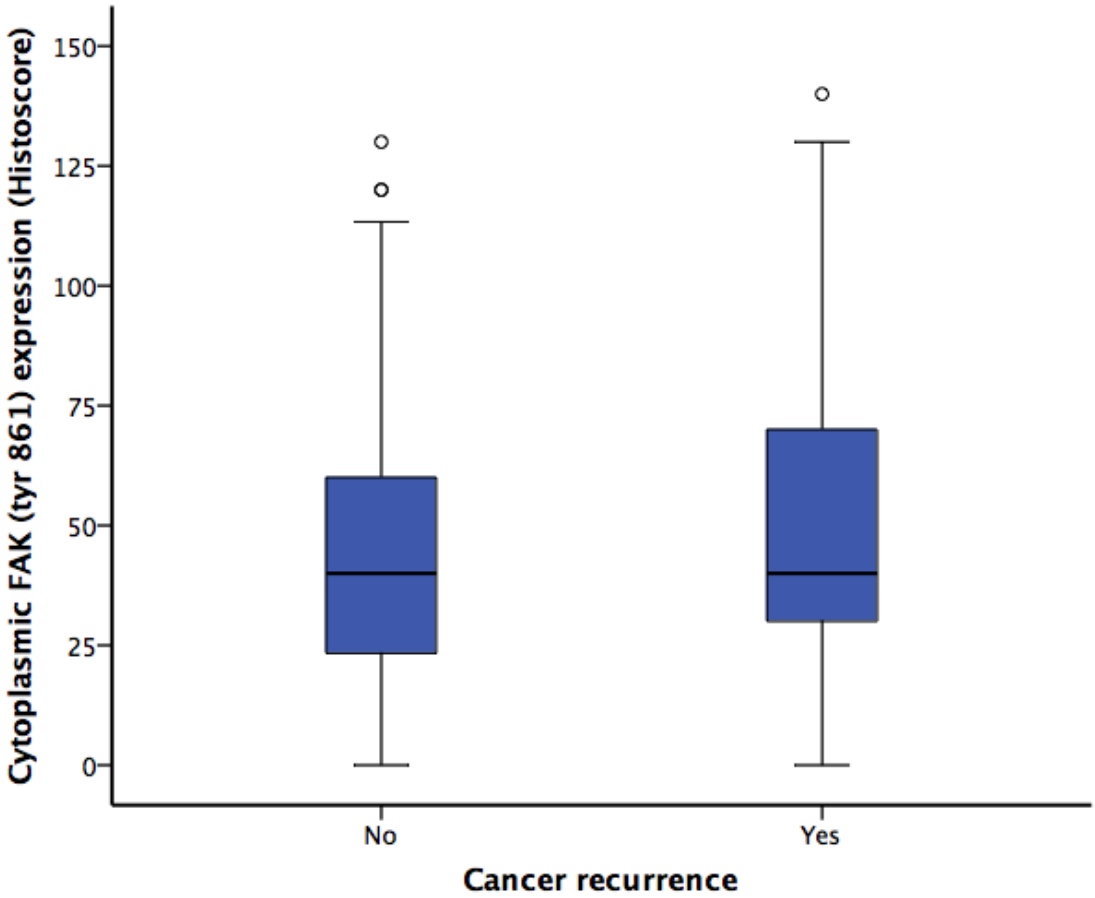
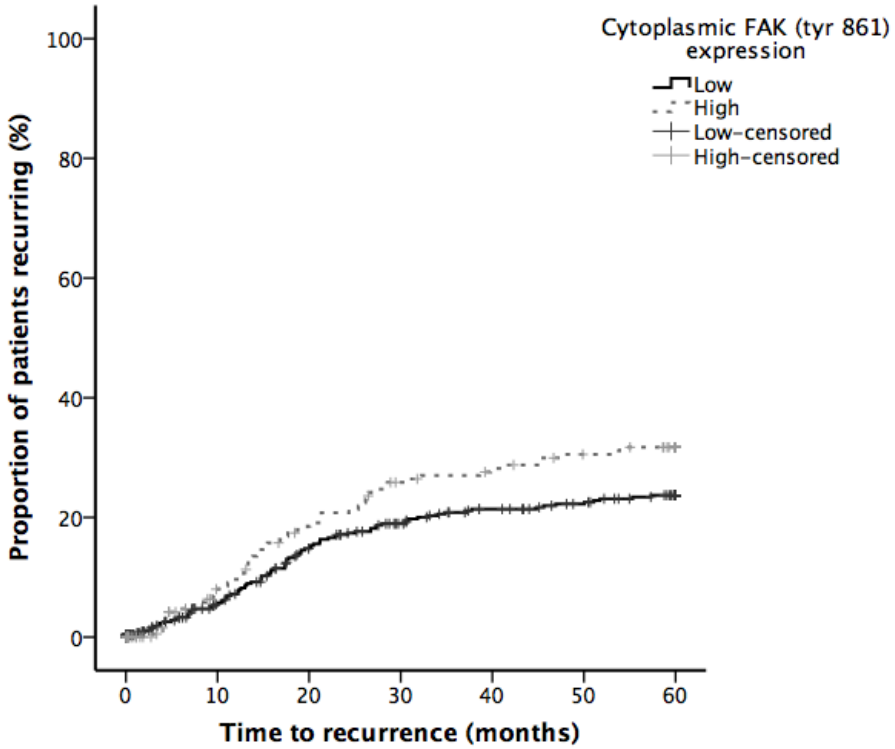


Figure 5.43: The distribution of cytoplasmic FAK (tyr 861) expression in patients with and without cancer recurrence (p=0.051)

The 5 year recurrence-free survival rate for patients with a high cytoplasmic FAK (tyr 861) expression was 51.4% compared to 54.2% in patients with a low cytoplasmic FAK (tyr 861) expression (p=0.502, Pearson's chi square). On logrank analysis raised cytoplasmic FAK (tyr 861) was associated with poor recurrence-free survival (p=0.046, figure 5.44). The mean survival for patients with a raised cytoplasmic FAK (tyr 861) was 47.3 months (95% CI 44.4 – 50.2) compared with 50.4 months (95% CI 48.6 – 52.2) in the low cytoplasmic FAK (tyr 861) group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	465	381	331	303	282	270	253
Low	212	167	145	129	123	116	109

Figure 5.44: The relationship between cytoplasmic FAK (tyr 861) expression and recurrence-free survival

Kaplan-Meier curve demonstrating the proportion of patients recurring stratified by cytoplasmic FAK (tyr 861) expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis raised FAK (tyr 861) was marginally associated with poor recurrence-free survival when dichotomised as a categorical variable (HR 1.39 (95% CI 1.00 – 1.94), p=0.047). The hazard ratio was also significant when it was included as a continuous variable (HR 1.01 (95% CI 1.00 – 1.01), p=0.048). Despite the associations observed between cytoplasmic FAK (tyr 861) expression and disease recurrence, its predictive value is relatively poor. When ROC analysis was performed using recurrence as the endpoint, the AUC was 0.55 (95% CI 0.50 – 0.60, p=0.051) for cytoplasmic FAK (tyr 861) as a continuous variable and an AUC of 0.54 (95% CI 0.49 – 0.60, p=0.108) for cytoplasmic FAK (tyr 861) as a categorical variable (figure 5.45).

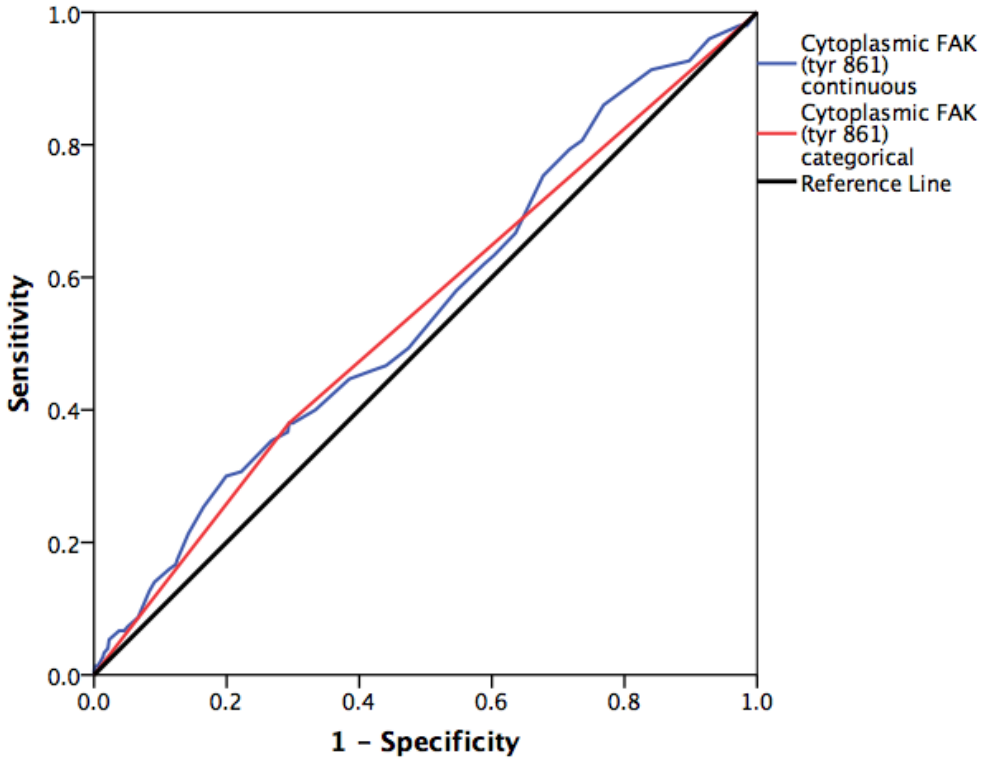


Figure 5.45: Predictive value of cytoplasmic FAK (tyr 861) expression in identifying patients who will develop cancer recurrence

Receiver-operator-characteristic curve demonstrating the predictive value of cytoplasmic FAK (tyr 861) in identifying patients who will develop cancer recurrence during follow-up.

5.4.6.2 Univariable overall survival - cytoplasmic FAK (tyr861) expression

Cytoplasmic FAK (tyr861) expression was not higher in patients who died during the follow up period (p=0.246, Mann-Whitney U). The median cytoplasmic FAK (tyr 861) was 40.00 (IQR 3.33 – 76.67) in the alive group compared to 40.00 (IQR 3.33 – 76.66) in patients who died during follow-up (supplementary figure 5.14, appendix 5.3).

The 5 year overall survival rate for patients with raised cytoplasmic FAK (tyr 861) was 55.2% compared to 58.5% in patients with a low cytoplasmic FAK (tyr 861) expression (p=0.420, Pearson's chi square). On logrank analysis raised cytoplasmic FAK (tyr 861) expression was not associated with poor overall survival (p=0.418). The mean survival for patients with raised cytoplasmic FAK (tyr 861) expression was 45.0 months (95% CI 42.0 – 48.0) compared with 46.2 months (95% CI 44.3 – 48.2) in the low cytoplasmic FAK (tyr 861) group (supplementary figure 5.15, appendix 5.3).

On Cox univariable regression analysis raised cytoplasmic FAK (tyr 861) expression was not associated with poor overall survival when dichotomised as a

categorical variable (HR 1.11 (95% CI 0.86 – 1.44), p=0.419) or as a continuous variable (HR 1.00 (95% CI 1.00 – 1.01), p=0.363). Furthermore, raised FAK (tyr 861) did not predict death when analysed as a continuous variable (AUC of 0.53 (95% CI 0.48 – 0.57, p=0.246)) or categorical variable (AUC of 0.51 (95% CI 0.47 – 0.56, p=0.531)) (supplementary figure 5.16, appendix 5.3).

5.4.6.3 Multivariable recurrence free survival

Proportionality assumptions testing

All the studied variables underwent Cox proportional hazards assumption testing as described in the statistical methodology section.

Cox proportional hazards regression analysis

All covariates with a significance level of p<0.1 were included in the multivariable model. Terms were removed if their respective p was >0.05. On univariable analysis, emergency presentation (p<0.001), advancing T-stage (p<0.001), advancing N-stage (p<0.001), higher TNM stage (p<0.001), poor differentiation (p=0.007), raised serum CRP (p=0.013), hypoalbuminaemia (p=0.011), poor Klintrup score (p<0.001) and tumour MSI (p=0.035), raised cytoplasmic HCK (p=0.004) and raised cytoplasmic FAK (tyr 861) (p=0.047) had a p-value <0.1 and were therefore included in the multivariable Cox proportional hazards regression model.

On multivariable analysis, advancing T-stage (when all subcategories were compared with T1 (T2 HR 0.61 (95% CI 0.12 – 3.15), (T3 HR 0.99 (95% CI 0.24 – 4.16) and (T4 HR 2.71 (95% CI 0.64 – 11.45)), p<0.001), N-stage (when all subcategories were compared with N0 (N1 HR 2.42 (95% CI 1.60 – 3.67), (N2 HR 3.24 (95% CI 1.96 – 5.34), p<0.001) good Klintrup score (HR 0.52 (95% CI 0.32 – 0.86), p=0.010) and raised cytoplasmic FAK (tyr 861) (HR 1.48 (95% CI (1.02 – 2.16), p=0.040) were independently and significantly associated with poor overall survival (table 5.6).

Table 5.6: The relationship between clinicopathological factors and recurrence free survival in patients with colorectal cancer: univariable and multivariable analysis

	Univariable analysis			Multivariable analysis		
	Coefficient	p-value	Hazard ratio (95% CI)	Coefficient	p-value	Hazard ratio (95% CI)
Age	-0.120	0.101	0.99 (0.98 - 1.00)			
Sex						
Female			1			
Male	0.121	0.461	1.13 (0.82 – 1.56)			
Presentation						
Elective			1			
Emergency	0.689	<0.001	1.99 (1.477 – 2.0)		0.091	
Tumour site						
Right	0.000	0.278	1			
Left	-0.249		0.78 (0.53 – 1.14)			
Rectum	0.085		1.09 (0.73 – 1.62)			
T stage						
1	0.000	<0.001	1	0.000	<0.001	1
2	-0.008		0.99 (0.21 – 4.77)	-0.494		0.61 (0.12 – 3.15)
3	1.010		2.75 (0.67 – 11.20)	-0.008		0.99 (0.24 – 4.16)
4	1.944		6.99 (1.71 – 28.49)	0.997		2.71 (0.64 – 11.45)
N stage						
0	0.000	<0.001	1	0.000	<0.001	1
1	1.133		3.12 (2.16 – 4.47)	0.885		2.42 (1.60 – 3.67)
2	1.702		5.49 (3.59 – 8.39)	1.175		3.24 (1.96 – 5.34)
TNM stage						
I	0.000	<0.001	1			
II	0.953		2.60 (1.18 – 5.70)			
III	2.051		7.78 (3.60 – 16.80)		0.841	
Differentiation						
Well/Moderate			1			
Poor	0.629	0.007	1.88 (1.18 – 2.98)		0.769	
Serum CRP						
Normal			1			
High	0.458	0.013	1.58 (1.10 – 2.27)		0.924	
Serum albumin						
Normal			1			
Low	0.495	0.011	1.64 (1.12 – 2.40)		0.240	
Klintrup score						
High			1			1
Low	-1.091	<0.001	0.34 (0.22 – 0.52)	-0.647	0.010	0.52 (0.32 – 0.86)
MSI status						
CI			1			
MSI	-0.689	0.035	0.50 (0.26 – 0.95)		0.548	
Cytoplasmic MMP-9						
Low			1			
High	-0.032	0.846	0.97 (0.70 – 1.33)			
Cytoplasmic FGR						
Low			1			
High	0.196	0.302	1.22 (0.84 – 1.76)			
Nuclear FGR						
Low			1			
High	0.029	0.878	1.03 (0.71 – 1.49)			
Cytoplasmic HCK						
Low			1			
High	0.496	0.004	1.64 (1.17 – 2.31)		0.394	
Cytoplasmic FAK (tyr 861)						
Low			1			1
High	0.334	0.047	1.40 (1.00 – 1.94)	0.395	0.040	1.48 (1.02 – 2.16)

5.4.6.4 Multivariable overall survival

On univariable survival analysis, none of the SFKs or FAK (tyr861) was associated with overall survival with all p-values >0.1. Therefore, no multivariable regression model was constructed based on these biomarkers.

5.4.7 The relationship between SFK expression, FAK (tyr 861) expression and TNM stage

5.4.7.1 FGR expression stratified by TNM stage II and III

There was no significant difference in the measurements of cytoplasmic FGR between patients with TNM stage II and III colorectal cancer (p=0.728, Mann-Whitney U, supplementary figure 5.17, appendix 5.3). The cytoplasmic FGR measurements in patients with stage II colorectal cancer ranged from 36.67 – 200.00 with a median of 110.00 (IQR 0.70 – 150.00) compared with a range of 40.00 – 170.00 and a median of 110.00 (IQR 76.67 – 143.34) in patients with stage III colorectal cancer. This observation was also supported by chi-squared analysis when cytoplasmic FGR was analysed as a categorical variable (p=0.190, Bonferroni adjustment p<0.0125, table 5.7).

Table 5.7 The relationship between SFK expression, FAK (tyr 861) expression and TNM stage II and III

Clinicopathological factors	TNM stage II	TNM stage III	p-value
Cytoplasmic FGR			
Low	245 (74.9%)	195 (79.6%)	0.190
High	82 (25.1%)	50 (20.4%)	
Nuclear FGR			
Low	257 (78.6%)	186 (75.9%)	0.449
High	70 (21.4%)	59 (24.1%)	
Cytoplasmic HCK			
Low	247 (75.5%)	176 (71.8%)	0.319
High	80 (24.5%)	69 (28.2%)	
Cytoplasmic FAK (tyr 861)			
Low	220 (67.3%)	169 (69.0%)	0.666
High	107 (32.7%)	76 (31.0%)	

Bonferoni adjustment = <0.0125

In addition to the observations above, there was no significant difference in the measurements of nuclear FGR between patients with TNM stage II and III colorectal cancer (p=0.640, Mann-Whitney U, supplementary figure 5.18, appendix 5.3). The nuclear FGR measurements in patients with stage II colorectal cancer ranged from 33.33 – 300.00 with a median of 165.00 (IQR 95.00 – 235.00) compared with a range of 60.00 – 300.00 and a median of 166.67 (IQR 91.67 – 241.67) in patients with stage III colorectal cancer. This observation was also supported by chi-squared analysis when nuclease FGR was analysed as a categorical variable (p=0.449, Bonferroni adjustment p<0.0125, table 5.7).

5.4.7.2 HCK expression stratified by TNM stage II and III colorectal cancer

There was no significant difference in the measurements of cytoplasmic HCK between patients with TNM stage II and III colorectal cancer (p=0.722, Mann-Whitney U, supplementary figure 5.19, appendix 5.3). Measurements of cytoplasmic HCK ranged from 0.00 – 110.00 with a median of 43.33 (IQR 16.66 – 70.00) in TNM stage II compared with a range of 0.00 – 103.33 and a median of 43.33 (IQR 13.33 – 73.33) in patients with TNM stage III. This observation was supported by chi-squared analysis when cytoplasmic HCK was analysed as a categorical variable (p=0.319, Bonferroni adjustment p<0.0125, table 5.7).

5.4.7.3 FAK (tyr 861) expression stratified by TNM stage II and III

There was no significant difference in the measurements of cytoplasmic FAK (tyr 861) between patients with TNM stage II and III colorectal cancer (p=0.435, Mann-Whitney U, supplementary figure 5.20, appendix 5.3). The cytoplasmic FAK (tyr 861) measurements in patients with stage II colorectal cancer ranged from 0.00 – 130.00 with a median of 43.33 (IQR 6.00 – 81.66) compared with a range of 0.00 – 120.00 and a median of 40.00 (IQR 3.33 – 76.67) in patients with stage III colorectal cancer. This observation was also supported by chi-squared analysis when cytoplasmic FAK (tyr 861) was analysed as a categorical variable (p=0.666, Bonferroni adjustment p<0.0125, table 5.7).

5.4.8 SFK expression and survival in patients with stage II colorectal cancer

During follow-up there were 54 (16.5%) recurrences and 114 (34.9%) deaths. Five year recurrence-free and overall survival rates for the clinicopathological factors studied can be found in appendix 3.3.

5.4.8.1 Univariable recurrence-free survival - cytoplasmic FGR expression

Cytoplasmic FGR measurements were not significantly different in patients who went on to develop disease recurrence (p=0.232, Mann-Whitney U, supplementary figure 5.21, appendix 5.3). The median for patients with recurrence was 103.33 (IQR 60.00 – 146.66) compared with 113.33 (IQR 74.99 – 151.67) in the non-recurrence group.

The 5 year recurrence-free survival rate for patients with a raised cytoplasmic FGR was 63.4% compared to 56.7% in patients with a low cytoplasmic FGR (p=0.288, Pearson's chi square). On logrank analysis raised cytoplasmic FGR was not significantly associated with poor recurrence-free survival (p=0.958). The mean survival for patients with a raised cytoplasmic FGR was 52.3 months (95% CI 48.6 – 56.0) compared with 52.9 months (95% CI 50.8 – 55.0) in the low cytoplasmic FGR group (supplementary figure 5.22, appendix 5.3).

Cox univariable regression analysis demonstrated that a raised cytoplasmic FGR expression was not significantly associated with poor recurrence-free survival when dichotomised as a categorical variable (HR 1.02 (95% CI 0.55 – 1.87), p=0.958) or as a continuous variable (HR 0.99 (95% CI 0.98 – 1.00), p=0.220). Furthermore, raised cytoplasmic FGR did not predict cancer recurrence as a continuous variable (AUC of 0.45 (95% CI 0.36 – 0.54, p=0.232)) or as a categorical variable (AUC of 0.51 (95% CI 0.42 – 0.59, p=0.906) (figure 5.46).

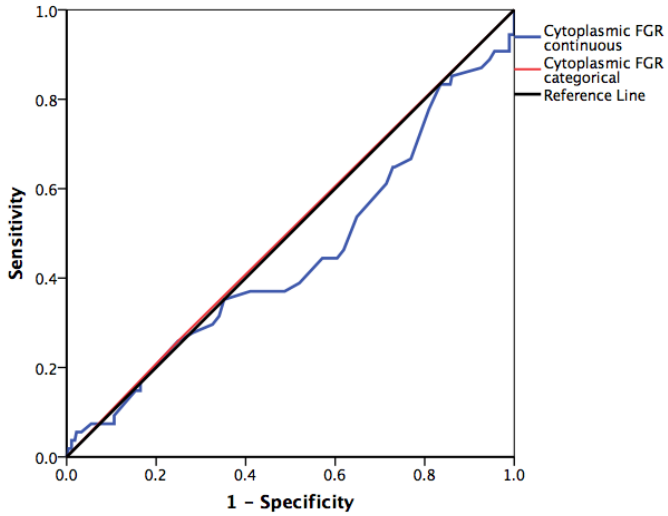


Figure 5.46: The predictive value of cytoplasmic FGR in identifying patients with stage II colorectal cancer who will develop recurrence during follow-up

Receiver-operator-characteristic curve demonstrating the predictive value of cytoplasmic FGR in identifying patients who will survive 5 years without recurrence.

5.4.8.2 Univariable recurrence-free survival - nuclear FGR expression

There was no difference between the nuclear FGR measurements of patients who did and did not develop cancer recurrence during follow-up (p=0.612, Mann-Whitney U). The median nuclear FGR was 166.67 (IQR 98.34 – 235.00) in the non-recurrence group compared to 160.00 (IQR 87.50 – 232.50) in the recurrence group (supplementary figure 5.23, appendix 5.3)

The 5 year recurrence-free survival rate for patients with a raised nuclear FGR was 54.3% compared to 59.5% in patients with a low nuclear FGR expression (p=0.430, Pearson's chi square). On logrank analysis raised nuclear FGR expression was not associated with poor recurrence-free survival (p=0.737). The mean survival for patients with raised nuclear FGR was 51.3 months (95% CI 46.8 – 55.8) compared with 53.1 months (95% CI 51.1 – 55.1) in the low nuclear FGR group (supplementary figure 5.24, appendix 5.3).

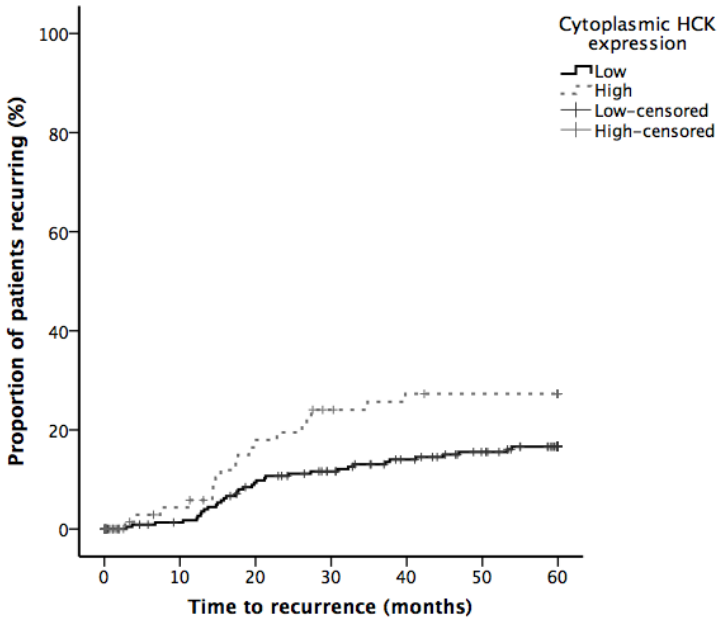
On Cox univariable regression analysis raised nuclear FGR was not significantly associated with poor recurrence-free survival when dichotomised as a categorical variable (HR 1.12 (95% CI 0.59 – 2.12), p=0.737) or as a continuous variable (HR 1.00 (95% CI 0.99 – 1.01), p=0.888). There was no statistically significant predictive association between nuclear FGR expression and disease recurrence when analysed using ROC analysis. With disease recurrence as the endpoint, the AUC was 0.48 (95% CI 0.39 – 0.57,

p=0.613) for nuclear FGR as a continuous variable and an AUC of 0.51 (95% CI 0.42 – 0.59, p=0.910) for nuclear FGR as a categorical variable (supplementary figure 5.25, appendix 5.3).

5.4.8.3 Univariable recurrence-free survival - cytoplasmic HCK expression

Cytoplasmic FGR measurements were not significantly different in patients who went on to develop disease recurrence (p=0.414, Mann-Whitney U). The median for patients with recurrence was 45.84 (IQR 5.84 – 85.84) compared with 43.33 (IQR 16.66 – 70.00) in the non-recurrence group (supplementary figure 5.26, appendix 5.3).

The 5 year recurrence-free survival rate for patients with a raised cytoplasmic HCK was 55.0% compared to 59.5% in patients with a low cytoplasmic HCK expression (p=0.476, Pearson's chi square). On logrank analysis raised cytoplasmic HCK was associated with poor recurrence-free survival (p=0.037, figure 5.47). The mean survival for patients with a raised cytoplasmic HCK was 48.8 months (95% CI 44.2 – 53.4) compared with 53.9 months (95% CI 52.0 – 55.8) in the low cytoplasmic HCK group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	247	222	201	188	174	162	148
High	80	65	55	48	45	44	44

Figure 5.47: The relationship between cytoplasmic HCK expression and recurrence free survival in patients with stage II colorectal cancer

Kaplan-Meier curve demonstrating the proportion of patients recurring stratified by cytoplasmic HCK expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

Cox univariable regression analysis demonstrated that a raised cytoplasmic HCK was significantly associated with poor recurrence-free survival when dichotomised as a categorical variable (HR 1.81 (95% CI 1.03 – 3.19), p=0.040) but not as a continuous variable (HR 1.01 (95% CI 1.00 – 1.02), p=0.132). Furthermore, raised cytoplasmic HCK did not predict cancer recurrence as a continuous variable (AUC of 0.54 (95% CI 0.44 – 0.63, p=0.415)) or as a categorical variable (AUC of 0.55 (95% CI 0.47 – 0.64, p=0.217) (figure 5.48).

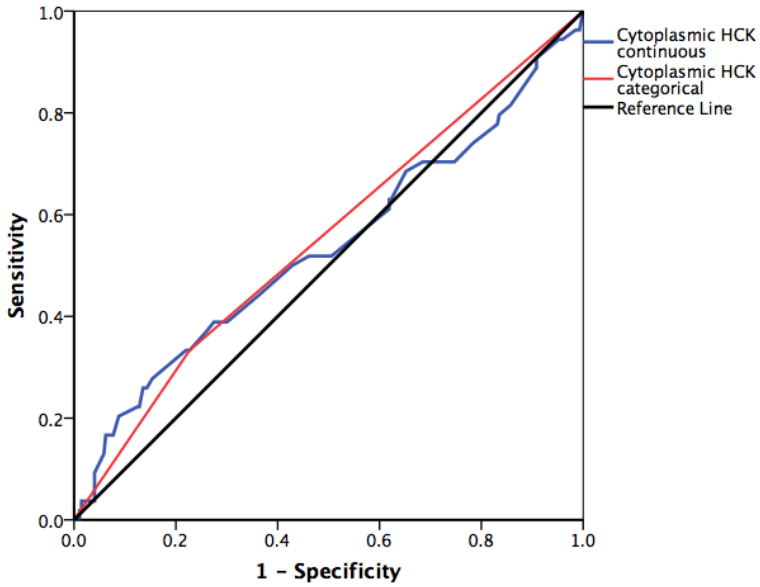


Figure 5.48: The predictive value of cytoplasmic HCK in identifying patients with stage II colorectal cancer who will develop recurrence during the follow up period

Receiver-operator-characteristic curve demonstrating the predictive value of cytoplasmic HCK in identifying patients who will survive 5 years without recurrence.

5.4.8.4 Univariable overall survival - cytoplasmic FGR expression

Cytoplasmic FGR measurements were not significantly higher in patients who died during follow-up (p=0.730, Mann-Whitney U, supplementary figure 5.27, appendix 5.3). The median for patients who died was 110.00 (IQR 73.33 – 146.67) compared with 113.33 (IQR 70.00 – 156.66) in the alive group.

The 5 year overall survival rate for patients with a raised cytoplasmic FGR was 67.1% compared to 58.8% in patients with a low cytoplasmic FGR expression (p=0.183, Pearson's chi square). On logrank analysis raised serum CRP was significantly associated with poor overall survival (p=0.621). The mean survival for patients with a raised cytoplasmic FGR expression was 48.7 months (95% CI 44.6 – 52.9) compared with 48.2 months (95% CI 45.7 – 50.7) in the low cytoplasmic FGR group (supplementary figure

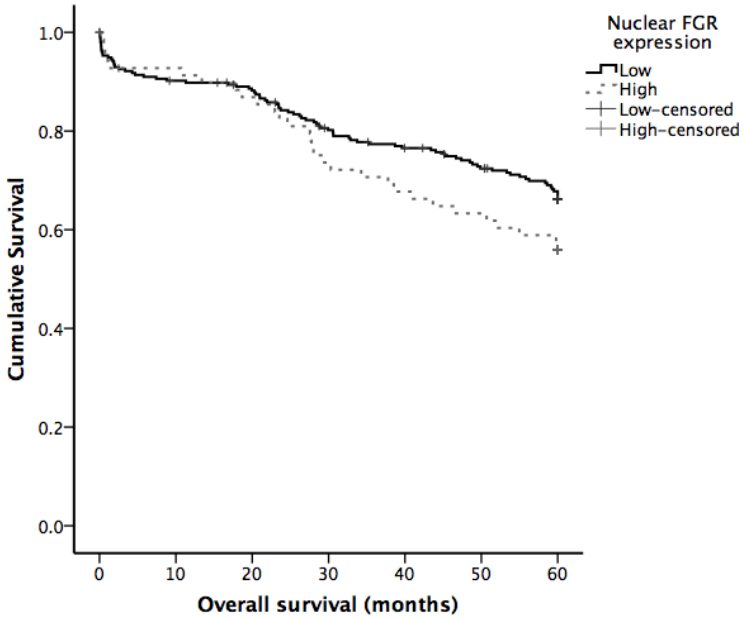
5.28, appendix 5.3).

On Cox univariable regression analysis raised cytoplasmic FGR was not associated with overall survival when dichotomised as a categorical variable (HR 0.90 (95% CI 0.58 – 1.38), p=0.621) or as a continuous variable (HR 1.00 95% CI 0.99 – 1.01, p=0.949). Furthermore, there was no predictive association between cytoplasmic FGR expression and survival when ROC analysis was performed using death as the endpoint. The AUC was 0.49 (95% CI 0.42 – 0.56, p=0.731) for cytoplasmic FGR as a continuous variable and an AUC of 0.49 (95% CI 0.42 – 0.56, p=0.750) for cytoplasmic FGR as a categorical variable (supplementary figure 5.29, appendix 5.3).

5.4.8.5 Univariable overall survival - nuclear FGR expression

Nuclear FGR expression measurements were not significantly different in patients who died during follow-up (p=0.460, Mann-Whitney U). The median nuclear FGR was 163.33 (IQR 96.66 – 230.00) in the alive group compared to 170.00 (IQR 83.34 – 256.66) in patients who did not survive 5 years (supplementary figure 5.30, appendix 5.3).

The 5 year overall survival rate for patients with a raised nuclear FGR expression was 54.3% compared to 62.6% in patients with a low nuclear FGR expression (p=0.204, Pearson's chi square). On logrank analysis raised nuclear FGR was not significantly associated with poor overall survival (p=0.125). The mean survival for patients with a raised nuclear FGR was 46.0 months (95% CI 41.4 – 50.7) compared with 49.0 months (95% CI 46.5 – 51.3) in the low nuclear FGR group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	257	229	221	198	187	175	161
Low	70	63	59	50	46	43	38

Figure 5.49: The relationship between nuclear FGR expression and overall survival in patients with stage II colorectal cancer

Kaplan-Meier curve demonstrating the proportion of patients surviving stratified by nuclear FGR expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

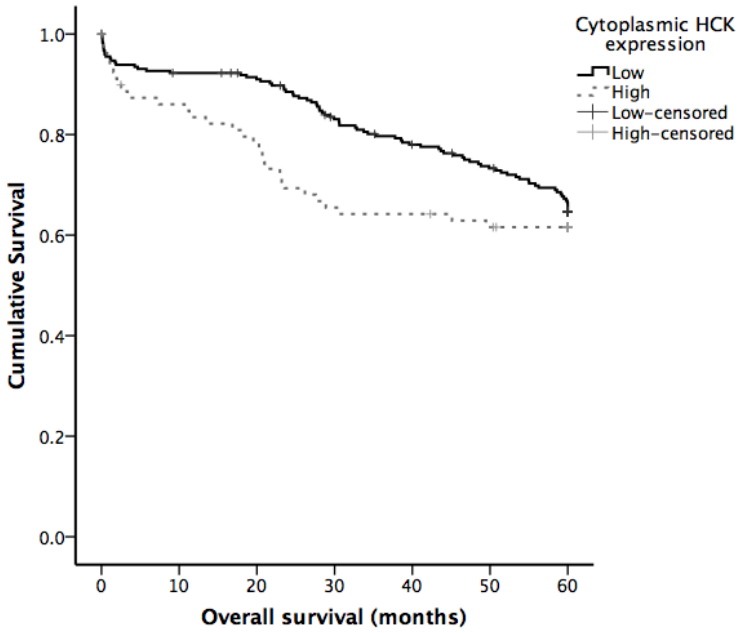
On Cox univariable regression analysis raised nuclear FGR was not with poor overall survival when dichotomised as a categorical variable (HR 1.38 (95% CI 0.91 – 2.10), p=0.126) or as a continuous variable (HR 1.00 (95% CI 1.00 – 1.01), p=0.385). When ROC analysis was performed using death as the endpoint, the AUC was 0.53 (95% CI 0.46 – 0.59, p=0.460) for nuclear FGR as a continuous variable and an AUC of 0.54 (95% CI 0.47 – 0.60, p=0.261) for nuclear FGR as a categorical variable (supplementary figure 5.31, appendix 5.3).

5.4.8.6 Univariable overall survival - cytoplasmic HCK expression

Cytoplasmic HCK measurements were not significantly higher in patients who died during follow-up (p=0.532, Mann-Whitney U). The median for patients who died was 40.00 (IQR 12.50 – 67.50) compared with 43.33 (IQR 16.66 – 70.00) in the alive group (supplementary figure 5.32, appendix 5.3).

The 5 year overall survival rate for patients with a raised cytoplasmic HCK was 56.2% compared to 62.3% in patients with a low cytoplasmic HCK expression (p=0.331,

Pearson's chi square). On logrank analysis raised cytoplasmic HCK was significantly associated with poor overall survival (p=0.260, figure 5.50). The mean survival for patients with a raised cytoplasmic HCK was 43.0 months (95% CI 37.9 – 48.1) compared with 50.0 months (95% CI 47.8 – 52.3) in the low cytoplasmic HCK group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	247	225	219	197	183	171	154
High	80	67	61	51	50	47	45

Figure 5.50: The relationship between cytoplasmic HCK expression and overall survival in patients with stage II colorectal cancer

Kaplan-Meier curve demonstrating the proportion of patients surviving stratified by cytoplasmic HCK expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis raised cytoplasmic HCK was not associated with poor overall survival when dichotomised as a categorical variable (HR 1.27 (95% CI 0.84 – 1.93), p=0.261) or as a continuous variable (HR 1.01 95% CI 1.00 – 1.02, p=0.124). Furthermore, cytoplasmic HCK was not predictive of death in follow-up. When ROC analysis was performed using death as the endpoint, the AUC was 0.52 (95% CI 0.46 – 0.59, p=0.533) for cytoplasmic HCK as a continuous variable and an AUC of 0.51 (95% CI 0.45 – 0.58, p=0.672) for cytoplasmic HCK as a categorical variable (supplementary figure 5.33, appendix 5.3).

5.4.8.7 Univariable recurrence free survival - cytoplasmic FAK (tyr 861)

There was no statistically significant difference between cytoplasmic FAK (tyr 861) measurements of patients who did and did not develop cancer recurrence during the follow up period (p=0.280, Mann-Whitney U). The median cytoplasmic FAK (tyr 861) was 43.33 (IQR 8.33 – 78.33) in the non-recurrence group compared to 46.67 (IQR 16.67 – 96.67) in the recurrence group (supplementary figure 5.34, appendix 5.3).

The 5 year recurrence free survival rate for patients with a raised cytoplasmic FAK (tyr 861) was 56.1% compared to 59.5% in patients with a low cytoplasmic FAK (tyr 861) expression (p=0.550, Pearson's chi square). On logrank analysis raised cytoplasmic FAK (tyr 861) was not associated with poor recurrence-free survival (p=0.263). The mean survival for patients with a raised cytoplasmic FAK (tyr 861) was 51.6 months (95% CI 48.2 – 55.0) compared with 53.3 months (95% CI 51.1 – 55.4) in the low cytoplasmic FAK (tyr 861) group (supplementary figure 5.35, appendix 5.3).

On Cox univariable regression analysis raised cytoplasmic FAK (tyr 861) was not significantly associated with poor recurrence-free survival when dichotomised as a categorical variable (HR 1.37 (95% CI 0.79 – 2.36), p=0.265) or as a continuous variable (HR 1.01 (95% CI 1.00 – 1.02), p=0.118). There was no statistically significant predictive association between cytoplasmic FAK (tyr 861) and disease recurrence when analysed using ROC analysis. With disease recurrence as the endpoint, the AUC was 0.55 (95% CI 0.46 – 0.64, p=0.280) for cytoplasmic FAK (tyr 861) as a continuous variable and an AUC of 0.54 (95% CI 0.45 – 0.62, p=0.391) for cytoplasmic FAK (tyr 861) as a categorical variable (supplementary figure 5.36, appendix 5.3).

5.4.8.8 Univariable overall survival - cytoplasmic FAK (tyr 861)

Cytoplasmic FAK (tyr 861) measurements were not significantly different in patients who died during follow-up (p=0.231, Mann-Whitney U). The median cytoplasmic FAK (tyr 861) was 43.33l (IQR 5.01 – 81.67) in the alive group compared to 44.17 (IQR 4.17 – 84.17) in patients who did not survive 5 years (supplementary figure 5.37, appendix 5.3).

The 5 year overall survival rate for patients with a raised cytoplasmic FAK (tyr 861) was 58.9% compared to 61.8% in patients with a low cytoplasmic FAK (tyr 861) expression (p=0.609, Pearson's chi square). On logrank analysis raised cytoplasmic FAK (tyr 861) was not associated with poor overall survival (p=0.475). The mean survival for patients with a raised cytoplasmic FAK (tyr 861) was 47.1 months (95% CI 43.1 – 51.1) compared with 48.9 months (95% CI 46.4 – 51.5) in the low cytoplasmic FAK (tyr 861)

group (supplementary figure 5.38, appendix 5.3).

On Cox univariable regression analysis raised cytoplasmic FAK (tyr 861) was not associated with poor overall survival when dichotomised as a categorical variable (HR 1.15 (95% CI 0.78 – 1.69), p=0.476) or when included as a continuous variable (HR 1.01 (95% CI 1.00 – 1.01), p=0.188).

Cytoplasmic FAK (tyr 861) was not predictive of death during follow-up. When ROC analysis was performed using death as the endpoint, the AUC was 0.54 (95% CI 0.48 – 0.61, p=0.232) for cytoplasmic FAK (tyr 861) as a continuous variable and an AUC of 0.52 (95% CI 0.45 – 0.58, p=0.588) for cytoplasmic FAK (tyr 861) as a categorical variable (Supplementary figure 5.39, appendix 5.3).

5.4.8.9 Multivariable recurrence free survival

Proportionality assumptions testing

All the studied variables underwent Cox proportional hazards assumption testing as described previously.

Cox proportional hazards regression analysis

All covariates with a significance level of p<0.1 were included in the multivariable model. Terms were removed if their respective p-values were >0.05. On univariable analysis, advancing age (p=0.096), emergency presentation (p=0.015), advancing T-stage (p<0.001), raised serum CRP (p=0.068), and tumour MSI status (p=0.092) and raised cytoplasmic HCK (p=0.040) had a p-value <0.1 and were therefore included in the multivariable Cox proportional hazards regression model.

On multivariable analysis, advancing T-stage (HR 2.99 (95% CI 1.65 – 5.40), p<0.045), emergency presentation (HR 1.88 (95% CI 1.04 – 3.40), p=0.038) and raised cytoplasmic HCK (HR 2.04 (95% CI (1.11 – 3.76), p=0.022) was independently and significantly associated with poor recurrence-free survival (table 5.8).

Table 5.8: The relationship between clinicopathological factors and recurrence-free survival in patients with stage II colorectal cancer – univariable and multivariable analysis

	Univariable analysis			Multivariable analysis		
	Coefficient	p-value	Hazard ratio (95% CI)	Coefficient	p-value	Hazard ratio (95% CI)
Age	-0.020	0.096	0.98 (0.96 - 1.00)		0.470	
Sex						
Female			1			
Male	0.243	0.376	1.28 (0.74 – 2.19)			
Presentation						
Elective			1			1
Emergency	0.671	0.015	1.96 (1.14 – 3.36)	0.629	0.038	1.88 (1.04 – 3.40)
Tumour site						
Right	0.000	0.533	1			
Left	0.250		1.28 (0.70 – 2.37)			
Rectum	0.373		1.45 (0.73 – 2.90)			
T stage						
1	N/A		N/A	N/A		N/A
2	N/A		N/A	N/A		N/A
3	(0.000)		1	(0.000)		1
4	0.973	<0.001	2.65 (1.55 – 4.51)	1.086	<0.001	2.96 (1.64 – 5.36)
Differentiation						
Well/Moderate			1			
Poor	0.037	0.938	1.04 (0.41 – 2.60)			
Serum CRP						
Normal			1			
High	0.561	0.068	1.75 (0.96 – 3.20)		0.357	
Serum albumin						
Normal			1			
Low	0.298	0.350	1.35 (0.72 – 2.52)			
Klintrup score						
High			1			
Low	-0.471	0.150	0.62 (0.33 – 1.19)			
MSI status						
CI			1			
MSI	-0.876	0.092	0.42 (0.15 – 1.15)		0.187	
Cytoplasmic MMP-9						
Low			1			
High	-0.191	0.483	0.83 (0.48 – 1.41)			
Cytoplasmic FGR						
Low			1			
High	0.016	0.958	1.02 (0.55 – 1.87)			
Nuclear FGR						
Low			1			
High	0.110	0.737	1.12 (0.59 – 2.12)			
Cytoplasmic HCK						
Low			1			1
High	0.593	0.040	1.81 (1.03 – 3.19)	0.713	0.022	2.04 (1.11 – 3.76)
Cytoplasmic FAK (tyr 861)						
Low			1			
High	0.311	0.265	1.37 (0.79 – 2.36)			

5.4.8.10 Multivariable overall survival

On univariable survival analysis, none of the SFKs or FAK (tyr861) was associated with overall survival with all p-values >0.1. Therefore, no multivariable regression model was constructed based on these biomarkers.

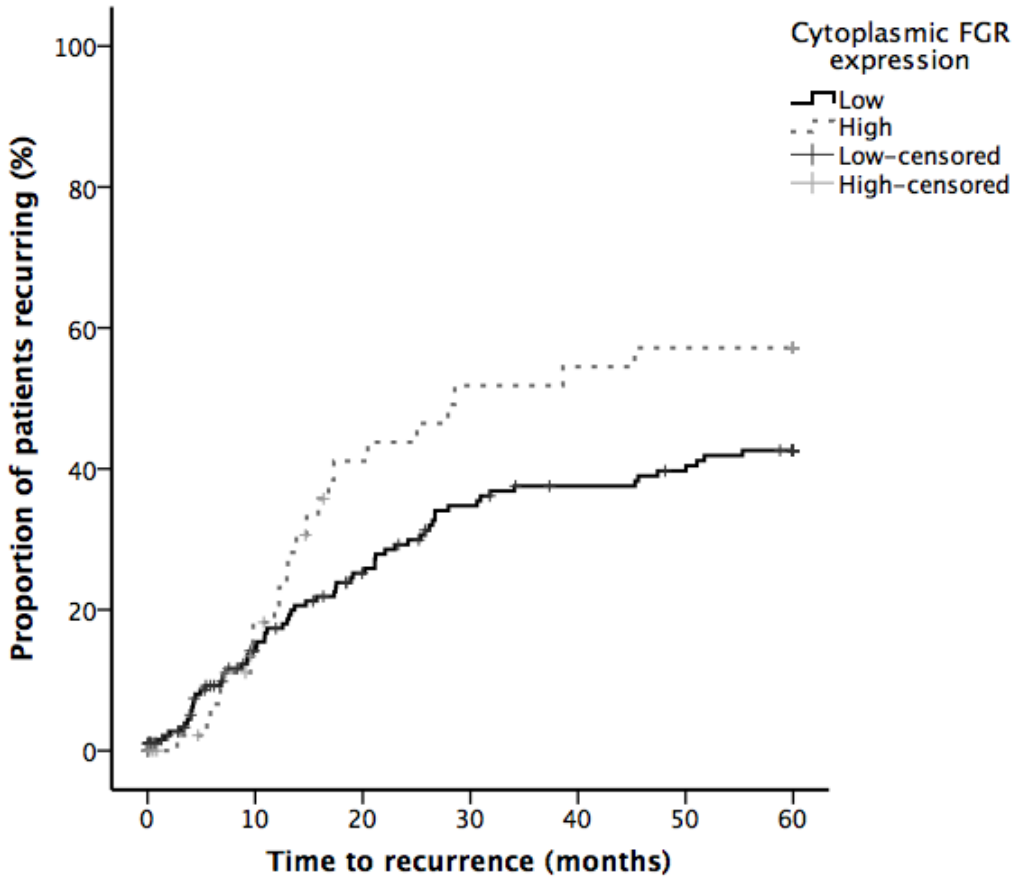
5.4.9 SFK expression and survival in patients with stage III colorectal cancer

During follow-up there were 89 (36.3%) recurrences and 121 (49.4%) deaths. Five year recurrence free and overall survival rates for the clinicopathological factors studied can be found in appendix 3.4.

5.4.9.1 Univariable recurrence-free survival - cytoplasmic FGR expression

Cytoplasmic FGR measurements were not significantly different in patients who went on to develop disease recurrence (p=0.609, Mann-Whitney U). The median for patients with recurrence was 110.00 (IQR 56.67 – 153.33) compared with 108.34 (IQR 75.00 – 142.67) in the non-recurrence group (Supplementary figure 5.40, appendix 5.3).

The 5 year recurrence-free survival rate for patients with a raised cytoplasmic FGR was 32.0% compared to 40.0% in patients with a low cytoplasmic FGR expression (p=0.299, Pearson's chi square). On logrank analysis raised cytoplasmic FGR was not significantly associated with poor recurrence-free survival (p=0.092, figure 5.51). The mean survival for patients with a raised cytoplasmic FGR was 35.5 months (95% CI 28.2 – 43.3) compared with 42.4 months (95% CI 38.9 – 45.9) in the low cytoplasmic FGR group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	195	134	111	94	87	83	78
High	50	34	22	18	17	16	16

Figure 5.51: The relationship between cytoplasmic FGR expression and recurrence-free survival in patients with stage III colorectal cancer

Kaplan-Meier curve demonstrating the proportion of patients recurring stratified by cytoplasmic FGR expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

Cox univariable regression analysis demonstrated that a raised cytoplasmic FGR expression was not significantly associated with poor recurrence-free survival when dichotomised as a categorical variable (HR 1.50 (95% CI 0.93 – 2.41), p=0.094) or as a continuous variable (HR 1.00 (95% CI 0.99 – 1.01), p=0.564). Furthermore, raised cytoplasmic FGR did not predict cancer recurrence as a continuous variable (AUC of 0.52 (95% CI 0.44 – 0.60, p=0.610)) or as a categorical variable (AUC of 0.54 (95% CI 0.47 – 0.62, p=0.267) (supplementary figure 5.41, appendix 5.3).

5.4.9.2 Univariable recurrence-free survival - nuclear FGR expression

There was no difference between the nuclear FGR measurements of patients who did and did not develop cancer recurrence during the follow up period (p=0.351, Mann-Whitney U). The median nuclear FGR was 166.67 (IQR 87.50 – 245.84) in the non-recurrence group compared to 166.67 (IQR 90.00 – 243.34) in the recurrence group (supplementary figure 5.42, appendix 5.3).

The 5 year recurrence-free survival rate for patients with a raised nuclear FGR was 45.8% compared to 36.0% in patients with a low nuclear FGR expression (p=0.180, Pearson's chi square). On logrank analysis raised nuclear FGR expression was not associated with poor recurrence-free survival (p=0.760). The mean survival for patients with raised nuclear FGR was 41.4 months (95% CI 35.4 – 47.5) compared with 40.8 months (95% CI 37.1 – 44.5) in the low nuclear FGR group (supplementary figure 5.43, appendix 5.3).

On Cox univariable regression analysis raised nuclear FGR was not significantly associated with poor recurrence-free survival when dichotomised as a categorical variable (HR 0.93 (95% CI 0.58 – 1.49), p=0.760) or as a continuous variable (HR 1.00 (95% CI 1.00 – 1.00), p=0.769). There was no statistically significant predictive association between nuclear FGR expression and disease recurrence when analysed using ROC analysis. With disease recurrence as the endpoint, the AUC was 0.54 (95% CI 0.46 – 0.61, p=0.351) for nuclear FGR as a continuous variable and an AUC of 0.51 (95% CI 0.44 – 0.59, p=0.719) for nuclear FGR as a categorical variable (supplementary figure 5.44, appendix 5.3).

5.4.9.3 Univariable recurrence-free survival - cytoplasmic HCK expression

Cytoplasmic HCK measurements were not significantly different in patients who went on to develop disease recurrence (p=0.065, Mann-Whitney U, figure 5.52). The median for patients with recurrence was 46.67 (IQR 15.00 – 78.34) compared with 41.67 (IQR 11.67 – 71.67) in the non-recurrence group.

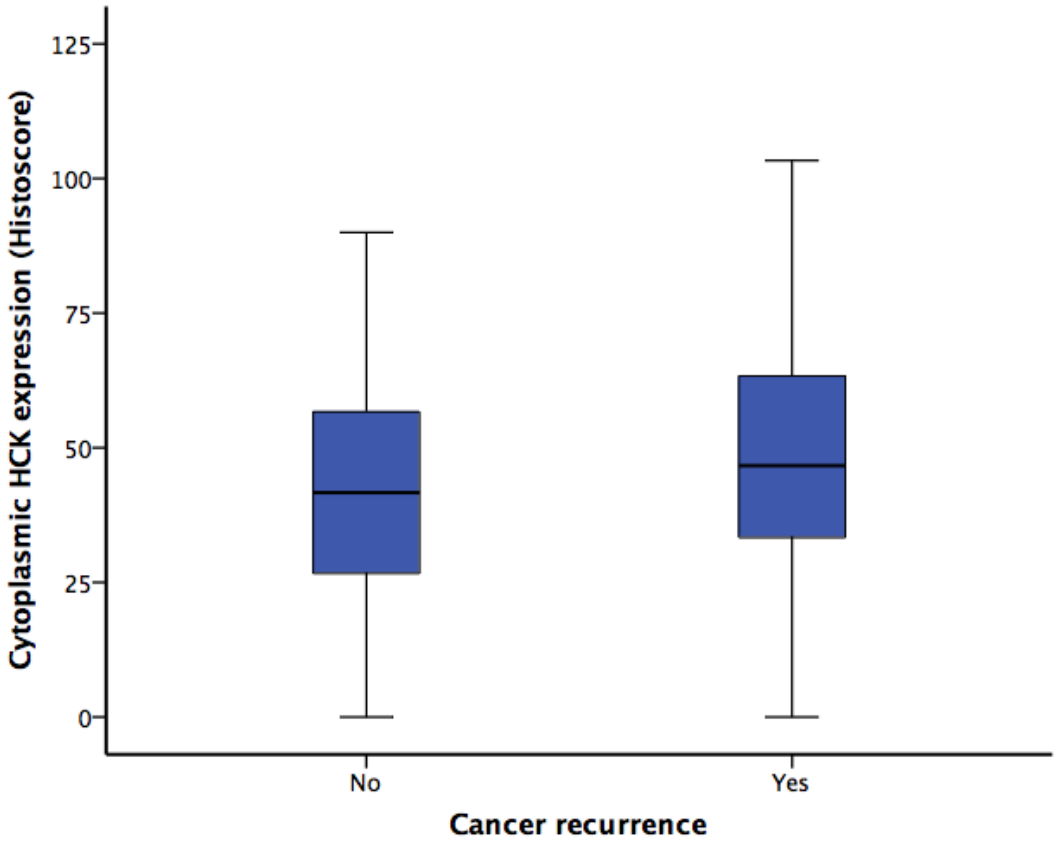
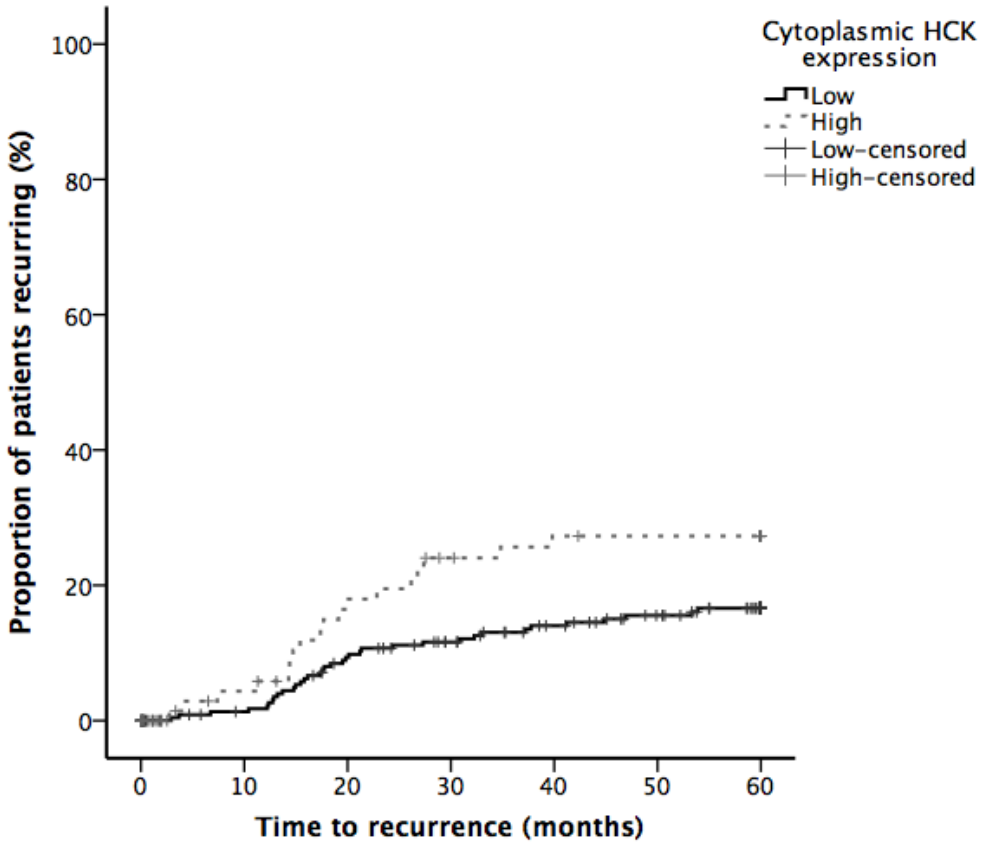


Figure 5.52: The distribution of cytoplasmic HCK measurements in patients with and without cancer recurrence in patients with stage III colorectal cancer (p=0.065)

The 5 year recurrence-free survival rate for patients with a raised cytoplasmic HCK was 36.2% compared to 39.2% in patients with a low cytoplasmic HCK expression (p=0.667, Pearson's chi square). On logrank analysis raised cytoplasmic HCK was not significantly associated with poor recurrence-free survival (p=0.324, figure 5.53). The mean survival for patients with a raised cytoplasmic HCK was 38.4 months (95% CI 32.8 – 44.0) compared with 42.2 months (95% CI 38.4 – 46.0) in the low cytoplasmic HCK group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	176	116	91	81	77	74	69
High	69	52	42	31	27	25	25

Figure 5.53: The relationship between cytoplasmic HCK expression and recurrence-free survival in patients with stage III colorectal cancer

Kaplan-Meier curve demonstrating the proportion of patients recurring stratified by cytoplasmic HCK expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

Cox univariable regression analysis demonstrated that a raised cytoplasmic HCK was not significantly associated with poor recurrence-free survival when dichotomised as a categorical variable (HR 1.25 (95% CI 0.81 – 1.93), p=0.325) but not as a continuous variable (HR 1.01 (95% CI 1.00 – 1.02), p=0.161). Furthermore, raised cytoplasmic HCK did not predict cancer recurrence as a continuous variable (AUC of 0.57 (95% CI 0.50 – 0.65, p=0.065)) or as a categorical variable (AUC of 0.55 (95% CI 0.48 – 0.63, p=0.173) (figure 5.54).

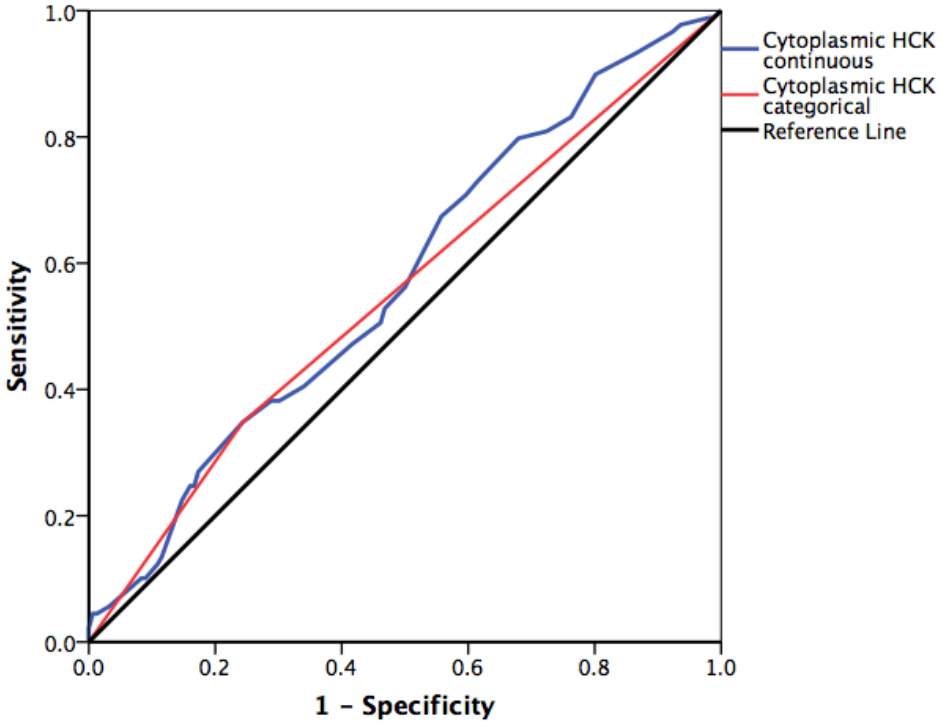


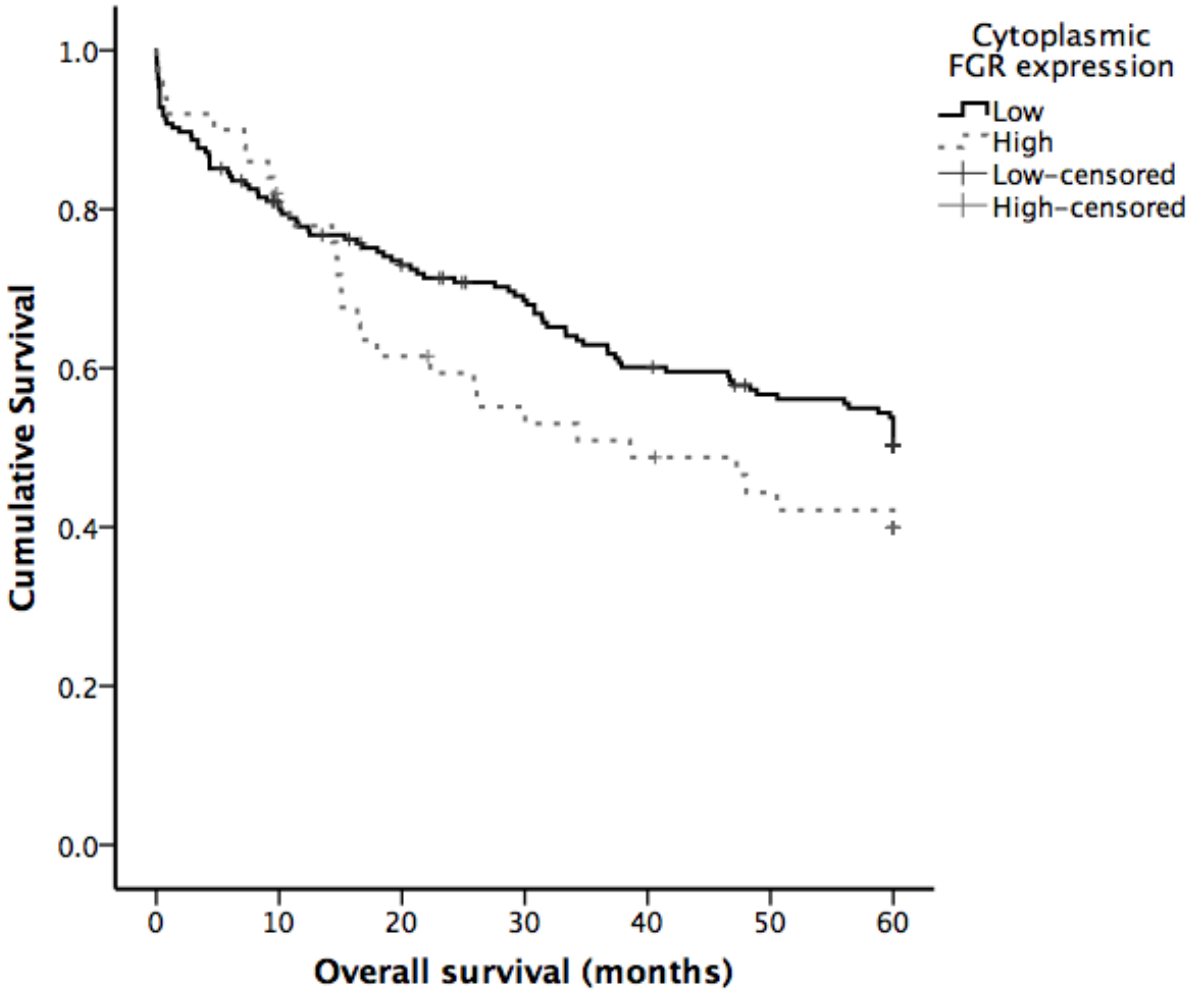
Figure 5.54: The predictive value of cytoplasmic HCK in identifying patients with stage III colorectal cancer who will develop recurrence during follow-up

Receiver-operator-characteristic curve demonstrating the predictive value of cytoplasmic HCK in identifying patients who will survive 5 years without recurrence.

5.4.9.4 Univariable overall survival - cytoplasmic FGR expression

Cytoplasmic FGR measurements were not significantly higher in patients who died during follow-up (p=0.494, Mann-Whitney U). The median for patients who died was 110.00 (IQR 70.00 – 150.00) compared with 106.67 (IQR 74.16 – 139.18) in the alive group (supplementary figure 5.45, appendix 5.3).

The 5 year overall survival rate for patients with a raised cytoplasmic FGR was 38.2% compared to 47.2% in patients with a low cytoplasmic FGR (p=0.245, Pearson's chi square). On logrank analysis raised cytoplasmic FGR was not significantly associated with poor overall survival (p=0.183, figure 5.55). The mean survival for patients with a raised cytoplasmic FGR expression was 36.0 months (95% CI 29.4 – 42.7) compared with 41.1 months (95% CI 37.8 – 44.5) in the low cytoplasmic FGR group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	195	152	134	122	107	98	93
High	50	40	30	26	23	20	19

Figure 5.55: The relationship between cytoplasmic FGR expression and overall survival in patients with stage III colorectal cancer

Kaplan-Meier curve demonstrating the proportion of patients surviving stratified by cytoplasmic FGR expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis raised cytoplasmic FGR was not associated with overall survival when dichotomised as a categorical variable (HR 1.33 (95% CI 0.87 – 2.01), p=0.185) or as a continuous variable (HR 1.00 95% CI 0.99 – 1.01, p=0.651). Furthermore, there was no predictive association between cytoplasmic FGR expression and survival when ROC analysis was performed using death as the endpoint. The AUC was 0.53 (95% CI 0.45 – 0.60, p=0.495) for cytoplasmic FGR as a continuous variable and an

AUC of 0.54 (95% CI 0.46 – 0.61, p=0.342) for cytoplasmic FGR as a categorical variable (supplementary figure 5.46, appendix 5.3).

5.4.9.5 Univariable overall survival - nuclear FGR expression

Nuclear FGR expression measurements were not significantly different in patients who died during follow-up (p=0.655, Mann-Whitney U). The median nuclear FGR was 173.33 (IQR 91.66 – 255.00) in the alive group compared to 163.33 (IQR 95.00 – 231.66) in patients who did not survive 5 years (Supplementary figure 5.47, appendix 5.3).

The 5 year overall survival rate for patients with a raised nuclear FGR was 47.5% compared to 44.6% in patients with a low nuclear FGR expression (p=0.703, Pearson's chi square). On logrank analysis raised nuclear FGR was not significantly associated with poor overall survival (p=0.499). The mean survival for patients with a raised nuclear FGR was 43.1 months (95% CI 37.4 – 48.8) compared with 39.1 months (95% CI 35.6 – 42.7) in the low nuclear FGR group (supplementary figure 5.48, appendix 5.3).

On Cox univariable regression analysis raised nuclear FGR was not associated with overall survival when dichotomised as a categorical variable (HR 0.87 (95% CI 0.57 – 1.32), p=0.500) or as a continuous variable (HR 1.00 (95% CI 1.00 – 1.01), p=0.459). When ROC analysis was performed using death as the endpoint, the AUC was 0.48 (95% CI 0.41 – 0.56, p=0.655) for nuclear FGR as a continuous variable and an AUC of 0.49 (95% CI 0.42 – 0.56, p=0.801) for nuclear FGR as a categorical variable (supplementary figure 5.49, appendix 5.3).

5.4.9.6 Univariable overall survival - cytoplasmic HCK expression

Cytoplasmic HCK measurements were not significantly higher in patients who died during follow-up (p=0.805, Mann-Whitney U). The median for patients who died was 45.00 (IQR 13.33 – 76.67) compared with 43.33 (IQR 13.33 – 73.33) in the alive group (supplementary figure 5.50, appendix 5.3).

The 5 year overall survival rate for patients with a raised cytoplasmic HCK was 46.4% compared to 44.9% in patients with a low cytoplasmic HCK expression (p=0.833, Pearson's chi square). On logrank analysis raised cytoplasmic HCK was not significantly associated with poor overall survival (p=0.830). The mean survival for patients with a raised cytoplasmic HCK was 41.7 months (95% CI 36.4 – 47.0) compared with 39.5 months (95% CI 35.8 – 43.1) in the low cytoplasmic HCK group (Supplementary figure 5.51, appendix 5.3).

On Cox univariable regression analysis raised cytoplasmic HCK was not associated with overall survival when dichotomised as a categorical variable (HR 0.96 (95% CI 0.65 – 1.42), p=0.831) or as a continuous variable (HR 1.00 (95% CI 0.99 – 1.01), p=0.966). When ROC analysis was performed using death as the endpoint, the AUC was 0.51 (95% CI 0.44 – 0.58, p=0.806) for cytoplasmic HCK as a continuous variable and an AUC of 0.51 (95% CI 0.44 – 0.58, p=0.839) for cytoplasmic HCK as a categorical variable (supplementary figure 5.52, appendix 5.3).

5.4.9.7 Univariable recurrence-free survival - cytoplasmic FAK (tyr 861)

Cytoplasmic FAK (tyr 861) measurements were significantly higher in patients who developed cancer recurrence during follow-up (p=0.029, Mann-Whitney U, figure 5.56). The median cytoplasmic FAK (tyr 861) measurement was 36.67 (IQR 0.00 – 76.67) in the non-recurrence group compared to 40.00 (IQR 1.67 – 78.34) in the recurrence group.

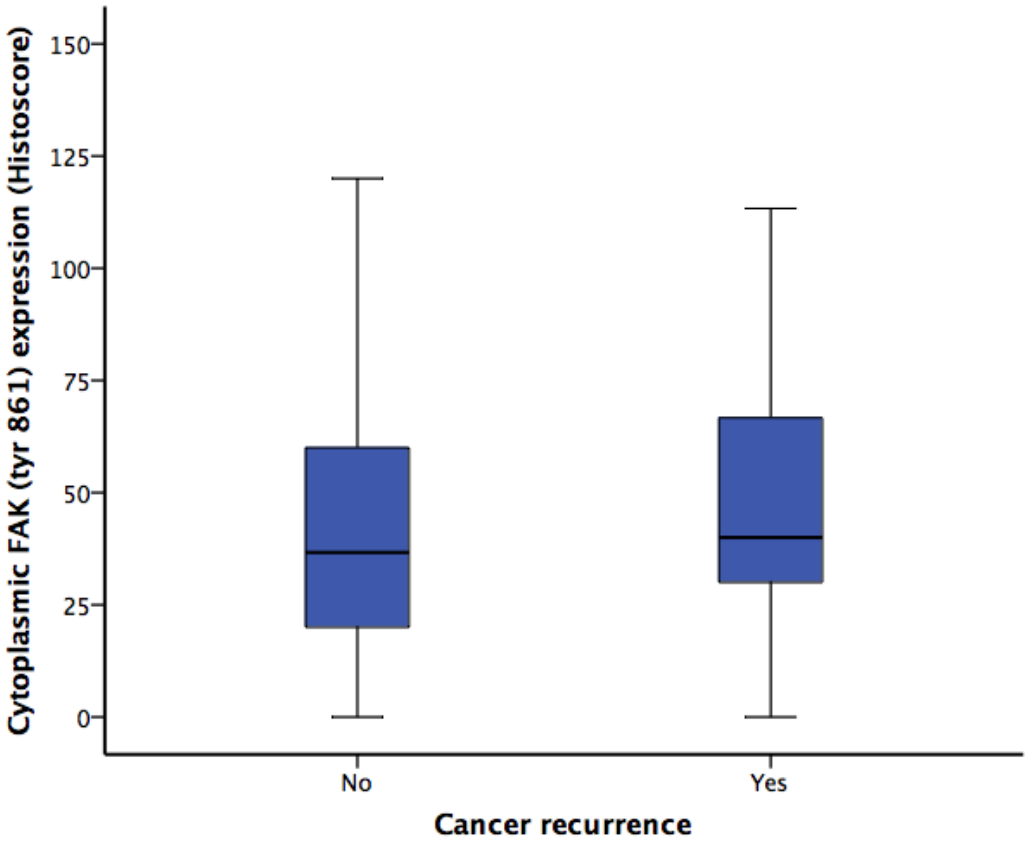
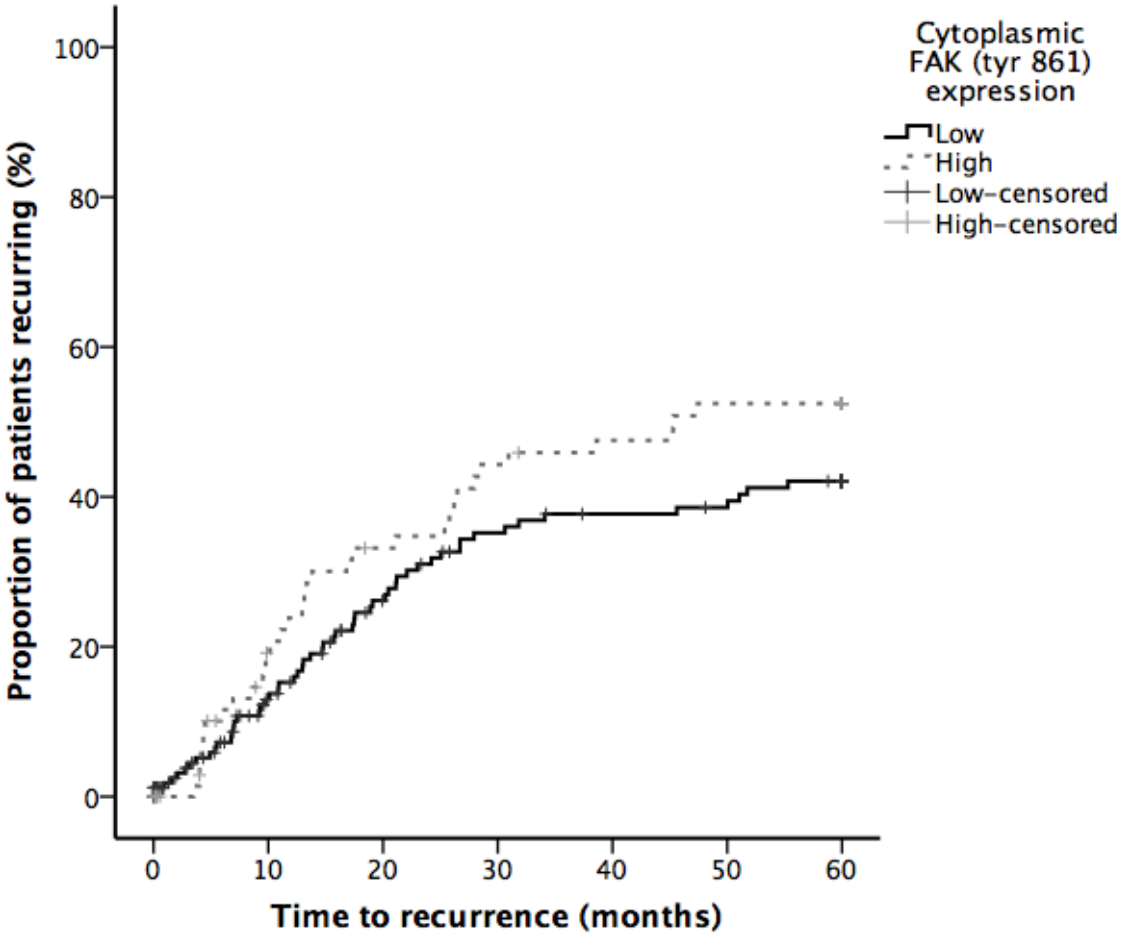


Figure 5.56: The distribution of cytoplasmic FAK (tyr 861) measurements in stage III colorectal cancer patients stratified by recurrence status (p=0.029)

The 5 year recurrence-free survival rate for patients with a raised cytoplasmic FAK (tyr 861) was 38.2% compared to 38.5% in patients with a low cytoplasmic FAK (tyr 861)

expression (p=0.964, Pearson's chi square). On logrank analysis raised cytoplasmic FAK (tyr 861) was not associated with poor recurrence-free survival (p=0.162, figure 5.57). The mean survival for patients with a raised cytoplasmic FAK (tyr 861) was 37.9 months (95% CI 32.3 – 43.4) compared with 42.5 months (95% CI 38.7 – 46.3) in the low cytoplasmic FAK (tyr 861) group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	169	116	91	77	72	70	65
Low	76	52	42	35	32	29	29

Figure 5.57: The relationship between cytoplasmic FAK (tyr 861) expression and recurrence-free survival in patients with stage III colorectal cancer

Kaplan-Meier curve demonstrating the proportion of patients recurring stratified by cytoplasmic FAK (tyr 861) expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis raised cytoplasmic FAK (tyr 861) was not significantly associated with poor recurrence-free survival when dichotomised as a categorical variable (HR 1.36 (95% CI 0.88 – 2.08), p=0.164) or as a continuous variable

(HR 1.00 (95% CI 1.00 – 1.01), p=0.374). Cytoplasmic FAK (tyr 861) was predictive of cancer recurrence when analysed using ROC analysis. With cancer recurrence as the endpoint, the AUC was 0.58 (95% CI 0.51 – 0.66, p=0.029) for cytoplasmic FAK (tyr 861) as a continuous variable and an AUC of 0.56 (95% CI 0.48 – 0.63, p=0.142) for cytoplasmic FAK (tyr 861) as a categorical variable (figure 5.58).

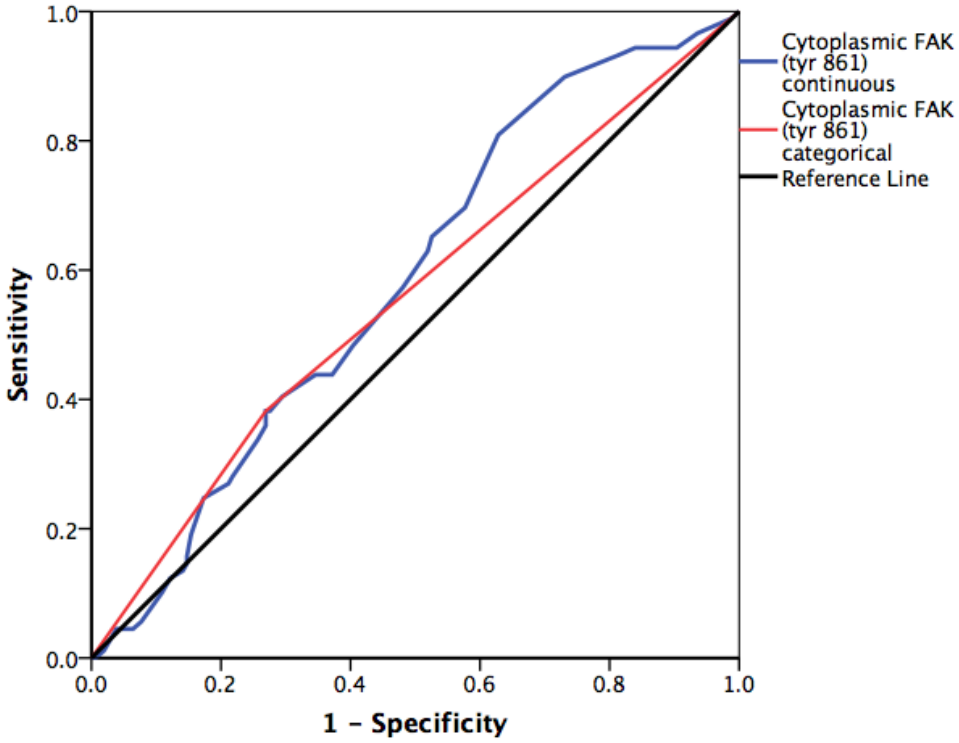


Figure 5.58: Predictive value of cytoplasmic FAK (tyr 861) in identifying patients with stage III colorectal cancer who will develop cancer recurrence

Receiver-operator-characteristic curve demonstrating the predictive value of cytoplasmic FAK (tyr 861) in identifying patients who will develop disease recurrence.

5.4.9.8 Univariable overall survival - cytoplasmic FAK (tyr 861)

Cytoplasmic FAK (tyr 861) measurements were not significantly different in patients who died during the follow up period (p=0.953, Mann-Whitney U). The median cytoplasmic FAK (tyr 861) was 40.00 (IQR 3.33 – 76.67) in the alive group compared to 36.67 (IQR 0.00 – 74.17) in patients who did not survive 5 years (supplementary figure 5.53, appendix 5.3).

The 5 year overall survival rate for patients with raised cytoplasmic FAK (tyr 861) was 44.7% compared to 45.6% in patients with a low cytoplasmic FAK (tyr 861) expression (p=0.904, Pearson's chi square). On logrank analysis raised cytoplasmic FAK

(tyr 861) was not associated with poor overall survival (p=0.780). The mean survival for patients with a raised cytoplasmic FAK (tyr 861) was 41.6 months (95% CI 36.4 – 46.9) compared with 39.4 months (95% CI 35.7 – 43.0) in the low cytoplasmic FAK (tyr 861) group (supplementary figure 5.54, appendix 5.3).

On Cox univariable regression analysis raised cytoplasmic FAK (tyr 861) was not associated with poor overall survival when dichotomised as a categorical variable (HR 0.95 (95% CI 0.54 – 1.39), p=0.780) or when included as a continuous variable (HR 1.00 (95% CI 0.99 – 1.00), p=0.494). Cytoplasmic FAK (tyr 861) was not predictive of death during the follow up period. When ROC analysis was performed using death as the endpoint, the AUC was 0.50 (95% CI 0.43 – 0.57, p=0.953) for cytoplasmic FAK (tyr 861) as a continuous variable and an AUC of 0.50 (95% CI 0.42 – 0.57, p=0.906) for cytoplasmic FAK (tyr 861) as a categorical variable (supplementary figure 5.55, appendix 5.3).

5.4.9.9 Multivariable recurrence-free survival

Proportionality assumptions testing

All the studied variables underwent Cox proportional hazards assumption testing.

Cox proportional hazards regression analysis

All covariates with a significance level of p<0.1 were included in the multivariable model. Terms were removed if their respective p-values were >0.05. On univariable analysis, emergency presentation (p=0.031), advancing T-stage (p=0.001), higher N stage (p=0.013), poor differentiation (p=0.026), raised serum CRP (p=0.095), hypoalbuminaemia (p=0.008), good Klintrup score (p=0.001) and cytoplasmic FGR (p=0.096) had p-values <0.1 and were therefore included in the model.

On multivariable analysis, advancing T-stage (when all subcategories were compared with T1 (T2 HR 281.25 (95% CI 0.00 – 8.52e45), (T3 HR 293.05 (95% CI 0.00 – 8.82e45) and (T4 HR 702.79 (95% CI 0.00 – 2.17e46)), p=0.012) and good Klintrup score (HR 0.40 (95% CI 0.18 – 0.91), p=0.029) were independently associated with poor recurrence free survival (table 5.9).

Table 5.9: The relationship between clinicopathological factors and recurrence-free survival in patients with stage III colorectal cancer – univariable and multivariable analysis

	Univariable analysis			Multivariable analysis		
	Coefficient	p-value	Hazard ratio (95% CI)	Coefficient	p-value	Hazard ratio (95% CI)
Age	-0.002	0.834	1.00 (0.98 - 1.02)			
Sex						
Female			1			
Male	-0.092	0.665	1.10 (0.72 – 1.66)			
Presentation						
Elective			1			
Emergency	0.472	0.031	1.60 (1.04 – 2.47)		0.983	
Tumour site						
Right	0.000	0.102	1			
Left	-0.496		0.61 (0.37 – 1.00)			
Rectum	0.065		1.07 (0.63 – 1.82)			
T stage						
1	(0.000)	0.001	1	(0.000)	0.012	1
2	5.972		392.11 (0.00 – 1.18e42)	5.639		281.25 (0.00 – 8.52e45)
3	6.852		945.66 (0.00 – 2.82e42)	5.680		293.05 (0.00 – 8.82e45)
4	7.666		2134.90 (0.00 – 6.37e42)	6.555		702.79 (0.00 – 2.11e46)
N stage						
1	(0.000)		1			
2	0.547	0.013	1.73 (1.12 – 2.69)		0.376	
Differentiation						
Well/Moderate			1			
Poor	0.617	0.026	1.85 (1.08 – 3.19)		0.399	
Serum CRP						
Normal			1			
High	0.407	0.095	1.50 (0.93 – 2.42)		0.431	
Serum albumin						
Normal			1			
Low	0.670	0.008	1.95 (1.20 – 3.20)		0.051	
Klintrup score						
High			1			1
Low	-1.257	0.001	0.28 (0.14 – 0.59)	-0.917	0.029	0.40 (0.18 – 0.91)
MSI status						
CI			1			
MSI	-0.096	0.836	0.91 (0.37 – 2.25)			
Cytoplasmic MMP-9						
Low			1			
High	0.096	0.652	1.10 (0.73 – 1.67)			
Cytoplasmic FGR						
Low			1			
High	0.406	0.094	1.50 (0.93 – 2.41)		0.134	
Nuclear FGR						
Low			1			
High	-0.074	0.760	0.93 (0.58 – 1.49)			
Cytoplasmic HCK						
Low			1			
High	0.219	0.325	1.25 (0.81 – 1.93)			
Cytoplasmic FAK (tyr 861)						
Low			1			
High	0.304	0.164	1.36 (0.88 – 2.08)			

5.4.9.10 Multivariable overall survival

On univariable survival analysis, none of the SFKs or FAK (tyr861) was associated with overall survival with all p-values >0.1. Therefore, no multivariable regression model was constructed based on these biomarkers.

5.4.10 The relationship between SFK expression, FAK (tyr 861) expression and MSI status

5.4.10.1 FGR expression stratified by MSI status

There was no significant difference in the measurements of cytoplasmic FGR between patients with CI and MSI colorectal cancer (p=0.567, Mann-Whitney U, supplementary figure 5.56, appendix 5.3). The cytoplasmic FGR measurements in patients with CI colorectal cancer ranged from 26.67 – 200.00 with a median of 110.00 (IQR 74.16 – 145.84) compared with a range of 66.67 – 210.00 and a median of 105.00 (IQR 78.33 – 141.67) in patients with MSI colorectal cancer. This observation was also supported by chi-squared analysis when cytoplasmic FGR was analysed as a categorical variable (p=0.822, Bonferroni adjustment p<0.0125, table 5.10).

Table 5.10: The relationship between SFK expression, FAK (tyr 861) expression and MSI status

Clinicopathological factors	CI	MSI	p-value
Cytoplasmic FGR			
Low	466 (78.7)	66 (77.6%)	0.822
High	126 (21.3%)	19 (22.4%)	
Nuclear FGR			
Low	453 (76.5%)	63 (74.1%)	0.627
High	139 (23.5%)	22 (25.9%)	
Cytoplasmic HCK			
Low	448 (75.7%)	65 (76.5%)	0.873
High	144 (24.3%)	20 (23.5%)	
Cytoplasmic FAK (tyr 861)			
Low	406 (68.6%)	59 (69.4%)	0.877
High	186 (31.4%)	26 (30.6%)	

Bonferoni adjustment <0.0125

In addition to the observations above, there was no significant difference in the measurements of nuclear FGR between patients with CI and MSI colorectal cancer (p=0.820, Mann-Whitney U, supplementary figure 5.57, appendix 5.3). The nuclear FGR measurements in patients with CI colorectal cancer ranged from 10.00 – 300.00 with a

median of 166.67 (IQR 90.00 – 243.34) compared with a range of 45.00 – 290.00 and a median of 170.00 (IQR 93.34 – 246.67) in patients with MSI colorectal cancer. This observation was also supported by chi-squared analysis when nuclear FGR was analysed as a categorical variable (p=0.627, Bonferroni adjustment p<0.0125, table 5.10).

5.4.10.2 HCK expression stratified by MSI status

There was no significant difference in the measurements of cytoplasmic HCK between patients with CI and MSI colorectal cancer (p=0.138, Mann-Whitney U, supplementary figure 5.58, appendix 5.3). Measurements of cytoplasmic HCK ranged from 0.00 – 110.00 with a median of 43.33 (IQR 16.66 – 70.00) in CI colorectal cancer compared with a range of 0.00 – 90.00 and a measurement of 40.00 (IQR 9.16 – 73.84) in patients with MSI colorectal cancer. This observation was supported by chi-squared analysis when cytoplasmic HCK was analysed as a categorical variable (p=0.873, Bonferroni adjustment p<0.0125, table 5.10).

5.4.10.3 FAK (tyr 861) expression stratified by MSI status

There was no significant difference in the measurements of cytoplasmic FAK (tyr 861) between patients with CI and MSI colorectal cancer (p=0.860, Mann-Whitney U, supplementary figure 5.59, appendix 5.3). The cytoplasmic FAK (tyr 861) measurements in patients with CI colorectal cancer ranged from 0.00 – 140.00 with a median of 40.00 (IQR 3.33 – 76.67) compared with a range of 0.00 – 120.00 and a median of 40.00 (IQR 3.34 – 76.66) in patients with MSI colorectal cancer. This observation was also supported by chi-squared analysis when cytoplasmic FAK (tyr 861) was analysed as a categorical variable (p=0.877, Bonferroni adjustment p<0.0125, table 5.10).

5.4.11 SFK expression and survival in patients with CI colorectal cancer

During follow-up there were 140 (23.6%) recurrences and 227 (38.3%) deaths. Five year recurrence free and overall survival rates for the clinicopathological factors studied can be found in appendix 3.5.

5.4.11.1 Univariable recurrence-free survival - cytoplasmic FGR expression

Cytoplasmic FGR measurements were not significantly different in patients who went on to develop disease recurrence (p=0.646, Mann-Whitney U, supplementary figure 5.60,

appendix 5.3). The median for patients with recurrence was 110.00 (IQR 70.00 – 150.00) compared with 110.00 (IQR 76.67 – 143.34) in the non-recurrence group.

The 5 year recurrence-free survival rate for patients with a raised cytoplasmic FGR was 56.3% compared to 52.8% in patients with a low cytoplasmic FGR expression (p=0.371, Pearson's chi square). On logrank analysis raised cytoplasmic FGR was not significantly associated with poor recurrence-free survival (p=0.540). The mean survival for patients with a raised cytoplasmic FGR was 47.4 months (95% CI 43.7 – 51.1) compared with 49.3 months (95% CI 47.4 – 51.2) in the low cytoplasmic FGR group (supplementary figure 5.61, appendix 5.3).

Cox univariable regression analysis demonstrated that a raised cytoplasmic FGR expression was not significantly associated with poor recurrence-free survival when dichotomised as a categorical variable (HR 1.13 (95% CI 0.77 – 1.67), p=0.540) or as a continuous variable (HR 1.00 (95% CI 0.99 – 1.01), p=0.625). Furthermore, raised cytoplasmic FGR did not predict cancer recurrence as a continuous variable (AUC of 0.49 (95% CI 0.43 – 0.54, p=0.647)) or as a categorical variable (AUC of 0.52 (95% CI 0.46 – 0.57, p=0.592) (supplementary figure 5.62, appendix 5.3).

5.4.11.2 Univariable recurrence free survival - nuclear FGR expression

There was no difference between the nuclear FGR measurements of patients who did and did not develop cancer recurrence during follow-up (p=0.715, Mann-Whitney U, supplementary figure 5.63, appendix 5.3). The median nuclear FGR was 170.00 (IQR 93.33 – 246.67) in the non-recurrence group compared to 166.67 (IQR 90.00 – 243.34) in the recurrence group.

The 5 year recurrence free survival rate for patients with a raised nuclear FGR was 54.0% compared to 53.4% in patients with a low nuclear FGR expression (p=0.912, Pearson's chi square). On logrank analysis raised nuclear FGR expression was not associated with recurrence-free survival (p=0.805). The mean survival for patients with raised nuclear FGR was 48.0 months (95% CI 44.5 – 51.6) compared with 49.1 months (95% CI 47.2 – 51.0) in the low nuclear FGR group (supplementary figure 5.64, appendix 5.3).

On Cox univariable regression analysis raised nuclear FGR was not significantly associated with recurrence-free survival when dichotomised as a categorical variable (HR 1.05 (95% CI 0.71 – 1.55), p=0.805) or as a continuous variable (HR 1.00 (95% CI 1.00 – 1.00), p=0.535). There was no statistically significant predictive association between

nuclear FGR expression and disease recurrence when analysed using ROC analysis. With disease recurrence as the endpoint, the AUC was 0.51 (95% CI 0.46 – 0.57, p=0.716) for nuclear FGR as a continuous variable and an AUC of 0.51 (95% CI 0.45 – 0.56, p=0.850) for nuclear FGR as a categorical variable (supplementary figure 5.65, appendix 5.3).

5.4.11.3 Univariable recurrence-free survival - cytoplasmic HCK expression

Cytoplasmic FGR measurements were not significantly different in patients who went on to develop disease recurrence (p=0.081, Mann-Whitney U, figure 5.59). The median for patients with recurrence was 45.00 (IQR 13.33 – 76.67) compared with 41.67 (IQR 11.67 – 71.67) in the non-recurrence group.

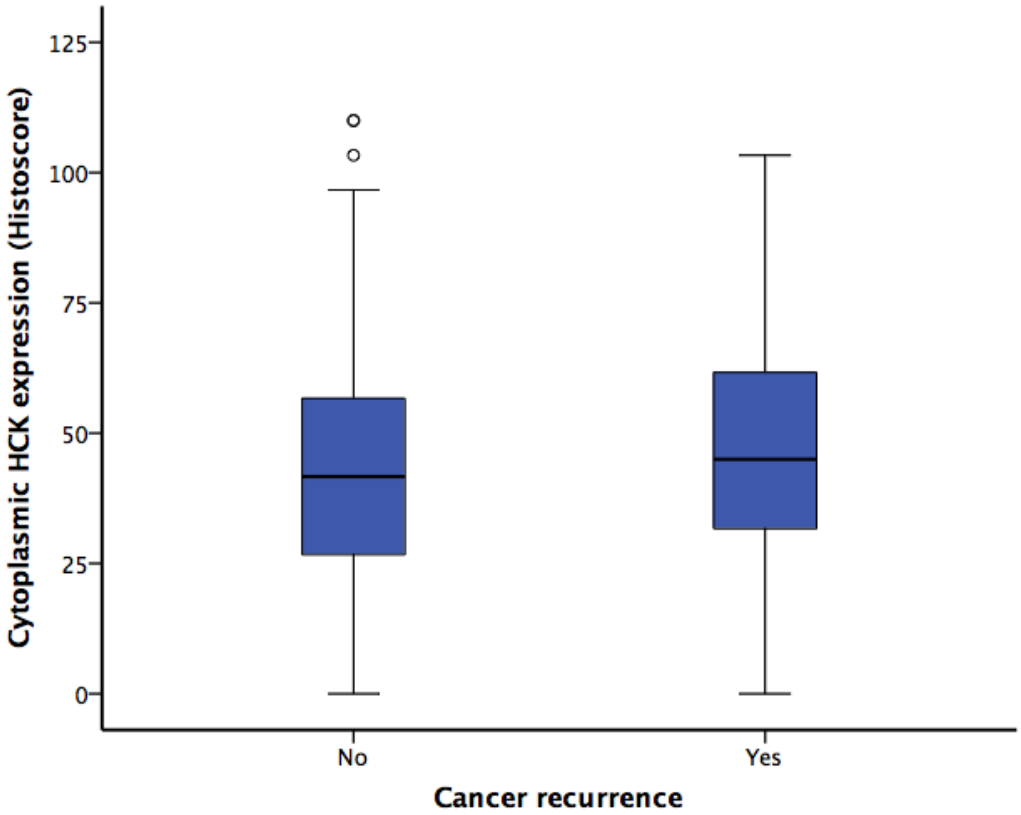
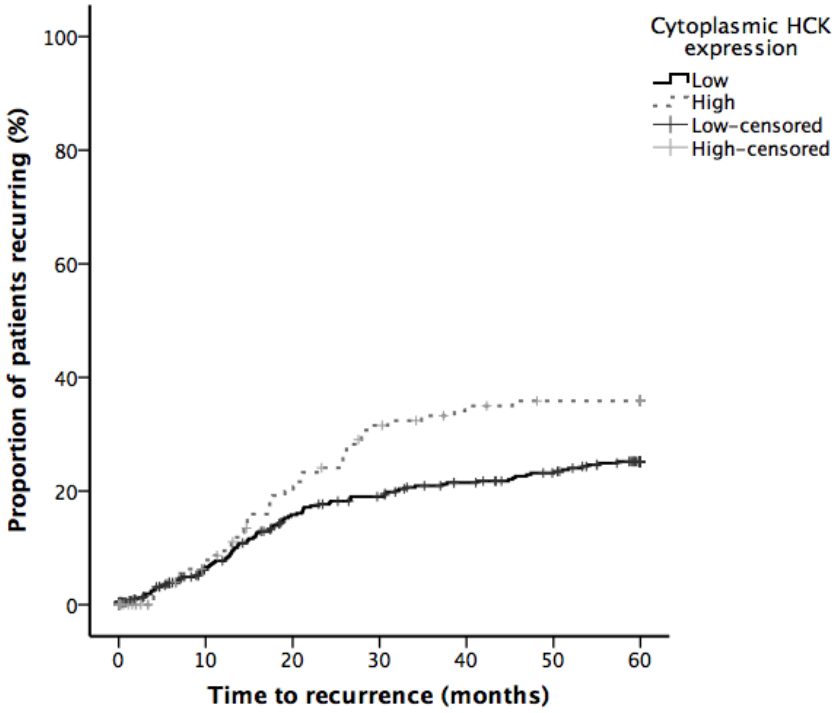


Figure 5.59: The distribution of cytoplasmic HCK measurements in patients with and without cancer recurrence in patients with CI colorectal cancer (p=0.081)

The 5 year recurrence-free survival rate for patients with a raised cytoplasmic HCK was 50.0% compared to 54.7% in patients with a low cytoplasmic HCK expression (p=0.327, Pearson's chi square). On logrank analysis raised cytoplasmic HCK was significantly associated with poor recurrence-free survival (p=0.022, figure 5.60). The mean survival for patients with a raised cytoplasmic HCK was 45.3 months (95% CI 41.7 – 48.9) compared with 50.0 months (95% CI 48.2 – 51.9) in the low cytoplasmic HCK group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	448	363	317	300	282	269	245
High	144	116	98	82	75	72	72

Figure 5.60: The relationship between cytoplasmic HCK expression and recurrence-free survival in patients with CI colorectal cancer

Kaplan-Meier curve demonstrating the proportion of patients recurring stratified by cytoplasmic HCK expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

Cox univariable regression analysis demonstrated that a raised cytoplasmic HCK was significantly associated with poor recurrence-free survival when dichotomised as a categorical variable (HR 1.51 (95% CI 1.06 – 2.16), p=0.023) and as a continuous variable (HR 1.01 (95% CI 1.00 – 1.02), p=0.027). Raised cytoplasmic HCK, however, did not predict cancer recurrence as a continuous variable (AUC of 0.55 (95% CI 0.49 – 0.60, p=0.081)) or as a categorical variable (AUC of 0.55 (95% CI 0.49 – 0.60, p=0.096) (figure 5.61).

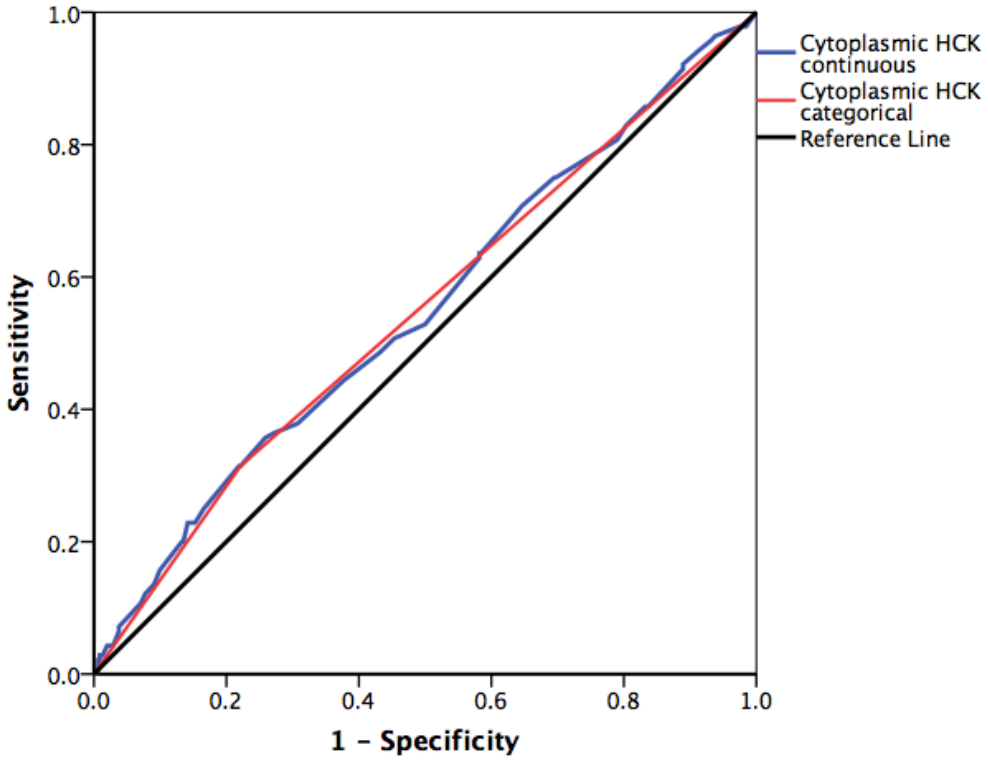


Figure 5.61: The predictive value of cytoplasmic HCK in identifying patients with CI colorectal cancer who will develop recurrence during follow-up

Receiver-operator-characteristic curve demonstrating the predictive value of cytoplasmic HCK in identifying patients who will survive 5 years without recurrence.

5.4.11.4 Univariable overall survival - cytoplasmic FGR expression

Cytoplasmic FGR measurements were not significantly higher in patients who died during follow-up (p=0.978, Mann-Whitney U, supplementary figure 5.66, appendix 5.3). The median for patients who died was 110.00 (IQR 60.00 – 150.00) compared with 110.00 (IQR 74.99 – 145.01) in the alive group.

The 5 year overall survival rate for patients with a raised cytoplasmic FGR was 60.0% compared to 57.1% in patients with a low cytoplasmic FGR (p=0.514, Pearson's chi square). On logrank analysis raised cytoplasmic FGR was not associated with poor overall survival (p=0.946). The mean survival for patients with a raised cytoplasmic FGR expression was 45.4 months (95% CI 41.7 – 49.2) compared with 46.4 months (95% CI 44.4 – 48.3) in the low cytoplasmic FGR group (supplementary figure 5.67, appendix 5.3).

On Cox univariable regression analysis raised cytoplasmic FGR was not associated with overall survival when dichotomised as a categorical variable (HR 1.01 (95% CI 0.74 – 1.39), p=0.947) or as a continuous variable (HR 1.00 95% CI 1.00 – 1.01, p=0.993).

Furthermore, there was no predictive association between cytoplasmic FGR expression and survival when ROC analysis was performed using death as the endpoint. The AUC was 0.50 (95% CI 0.45 – 0.55, p=0.978) for cytoplasmic FGR as a continuous variable and an AUC of 0.50 (95% CI 0.46 – 0.55, p=0.920) for cytoplasmic FGR as a categorical variable (supplementary figure 5.68, appendix 5.3).

5.4.11.5 Univariable overall survival - nuclear FGR expression

Nuclear FGR expression measurements were not significantly different in patients who died during follow-up (p=0.670, Mann-Whitney U, supplementary figure 5.69, appendix 5.3). The median nuclear FGR was 166.66 (IQR 100.00 – 233.32) in the alive group compared to 170.00 (IQR 90.00 – 250.00) in patients who did not survive 5 years.

The 5 year overall survival rate for patients with a raised nuclear FGR was 54.7% compared to 58.7% in patients with a low nuclear FGR expression (p=0.399, Pearson's chi square). On logrank analysis raised nuclear FGR was not associated with overall survival (p=0.312). The mean survival for patients with a raised nuclear FGR was 44.9 months (95% CI 41.3 – 48.5) compared with 46.5 months (95% CI 44.6 – 48.5) in the low nuclear FGR group (supplementary figure 5.70, appendix 5.3).

On Cox univariable regression analysis raised nuclear FGR was not associated with overall survival when dichotomised as a categorical variable (HR 1.17 (95% CI 0.87 – 1.57), p=0.126) or as a continuous variable (HR 1.00 (95% CI 1.00 – 1.00), p=0.653). When ROC analysis was performed using death as the endpoint, the AUC was 0.51 (95% CI 0.46 – 0.56, p=0.670) for nuclear FGR as a continuous variable and an AUC of 0.52 (95% CI 0.47 – 0.57, p=0.404) for nuclear FGR as a categorical variable (supplementary figure 5.71, appendix 5.3).

5.4.11.6 Univariable overall survival - cytoplasmic HCK expression

Cytoplasmic HCK measurements were not significantly higher in patients who died during follow-up (p=0.295, Mann-Whitney U, supplementary figure 5.72, appendix 5.3). The median was 43.33 (IQR 13.33 – 73.33) in both the alive and dead groups.

The 5 year overall survival rate for patients with a raised cytoplasmic HCK was 54.2% compared to 58.9% in patients with a low cytoplasmic HCK expression (p=0.314, Pearson's chi square). On logrank analysis raised cytoplasmic HCK was not associated with overall survival (p=0.254). The mean survival for patients with a raised cytoplasmic HCK was 43.3 months (95% CI 39.6 – 47.0) compared with 47.1 months (95% CI 45.1 –

49.0) in the low cytoplasmic HCK group (supplementary figure 5.73, appendix 5.3).

On Cox univariable regression analysis raised cytoplasmic HCK was not associated with overall survival when dichotomised as a categorical variable (HR 1.19 (95% CI 0.88 – 1.59), p=0.255) or as a continuous variable (HR 1.01 95% CI 1.00 – 1.01, p=0.142). Furthermore, cytoplasmic HCK was not predictive of death in the follow up period. When ROC analysis was performed using death as the endpoint, the AUC was 0.53 (95% CI 0.48 – 0.57, p=0.296) for cytoplasmic HCK as a continuous variable and an AUC of 0.52 (95% CI 0.47 – 0.57, p=0.484) for cytoplasmic HCK as a categorical variable (supplementary figure 5.74, appendix 5.3).

5.4.11.7 Univariable recurrence-free survival – cytoplasmic FAK (tyr861)

Cytoplasmic FAK (tyr 861) measurements were significantly higher in patients who developed cancer recurrence during follow-up (p=0.019, Mann-Whitney U, figure 5.62). The median cytoplasmic FAK (tyr 861) measurement was 40.00 (IQR 3.33 – 76.67) in the non-recurrence group compared to 41.67 (IQR 1.67 – 81.67) in the recurrence group.

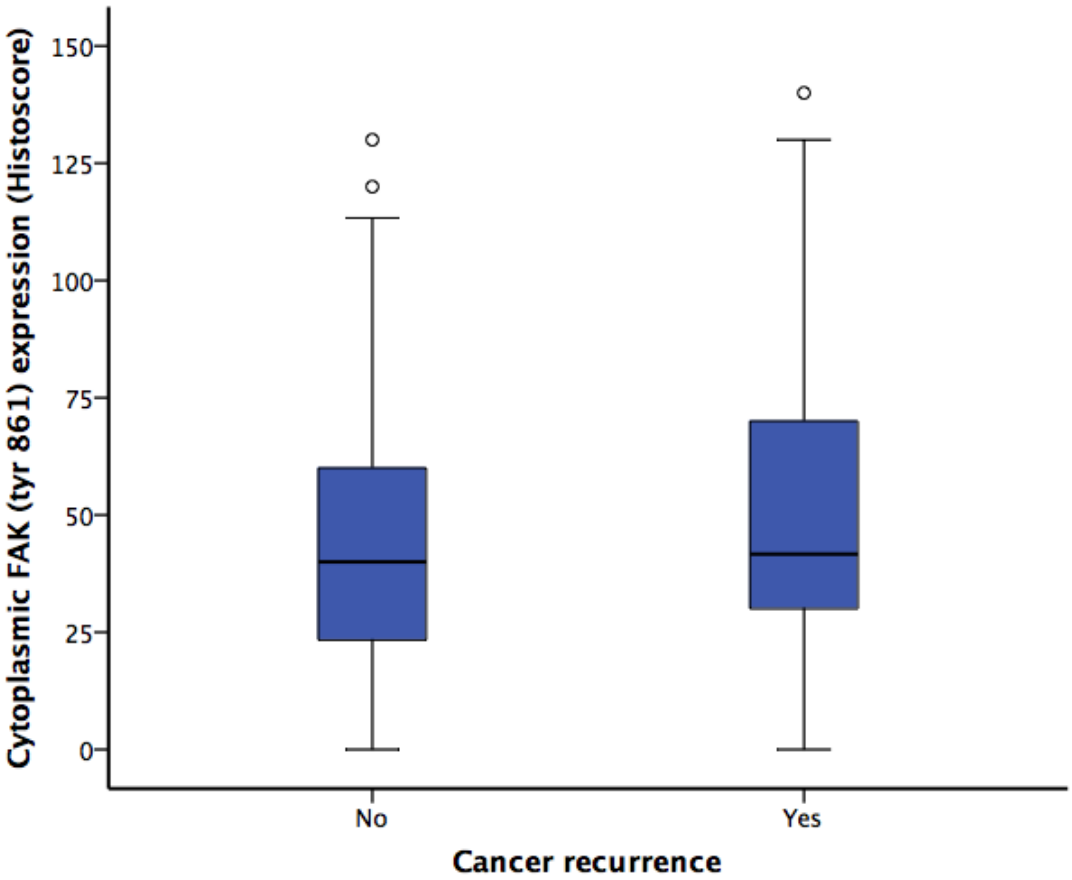
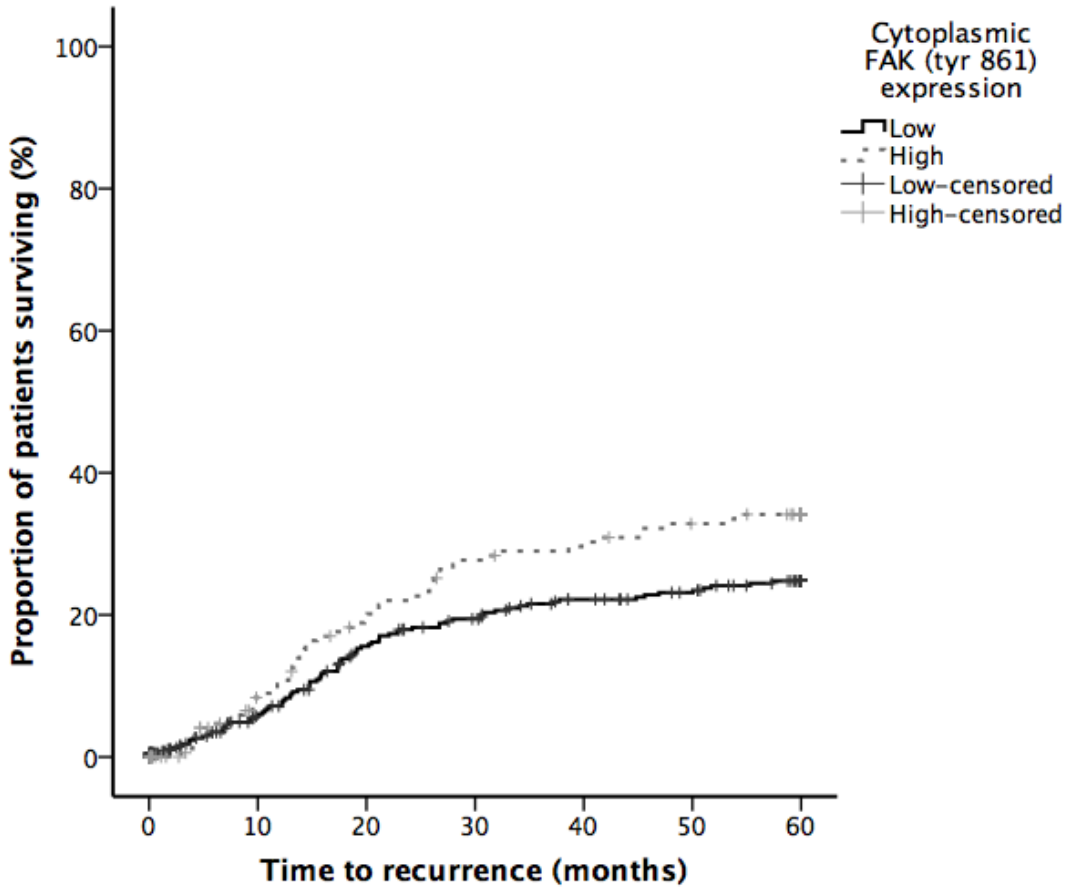


Figure 5.62: The distribution of cytoplasmic FAK (tyr 861) measurements in CI colorectal cancers stratified by recurrence status (p=0.019)

The 5 year recurrence-free survival rate for patients with a raised cytoplasmic FAK (tyr 861) was 52.2% compared to 54.2% in patients with a low FAK (tyr 861) expression (p=0.645, Pearson's chi square). On logrank analysis raised cytoplasmic FAK (tyr 861) was not associated with poor recurrence free survival (p=0.031, figure 5.63). The mean survival for patients with a raised cytoplasmic FAK (tyr 861) was 46.4 months (95% CI 43.3 – 49.5) compared with 53.0 months (95% CI 48.1 – 52.0) in the low cytoplasmic FAK (tyr 861) group.



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	406	330	287	268	248	238	220
Low	186	149	128	114	109	103	97

Figure 5.63: The relationship between cytoplasmic FAK (tyr 861) expression and recurrence-free survival in patients with CI colorectal cancer

Kaplan-Meier curve demonstrating the proportion of patients recurring stratified by cytoplasmic FAK (tyr 861) expression status. Beneath, there is a survival table demonstrating the number of patients not censored at each follow-up point.

On Cox univariable regression analysis raised cytoplasmic FAK (tyr 861) was significantly associated with poor recurrence-free survival when dichotomised as a categorical variable

(HR 1.45 (95% CI 1.03 – 2.03), p=0.032) and as a continuous variable (HR 1.01 (95% CI 1.00 – 1.01), p=0.025). Cytoplasmic FAK (tyr 861) predicted cancer recurrence when analysed using ROC analysis. With cancer recurrence as the endpoint, the AUC was 0.57 (95% CI 0.51 – 0.62, p=0.019) for cytoplasmic FAK (tyr 861) as a continuous variable and an AUC of 0.55 (95% CI 0.50 – 0.61, p=0.065) for cytoplasmic FAK (tyr 861) as a categorical variable (figure 5.64).

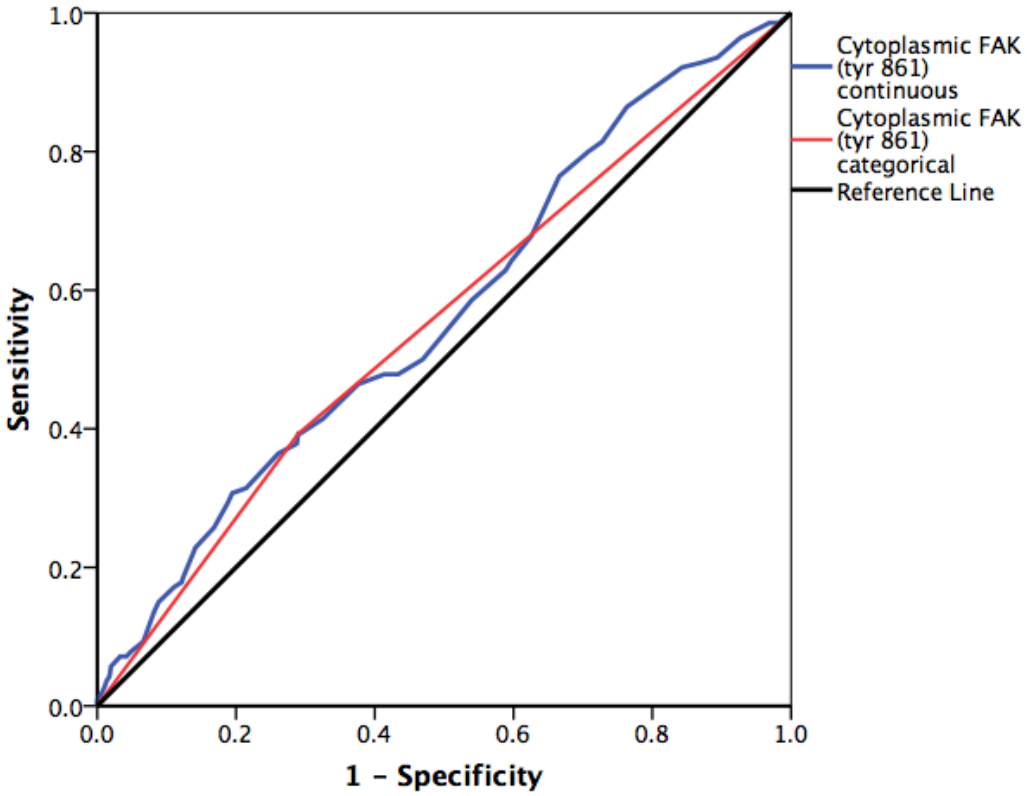


Figure 5.64: Predictive value of cytoplasmic FAK (tyr 861) in identifying patients with CI colorectal cancer who will develop cancer recurrence

Receiver-operator-characteristic curve demonstrating the predictive value of cytoplasmic FAK (tyr 861) in identifying patients who will develop disease recurrence.

5.4.11.8 Univariable overall survival - cytoplasmic FAK (tyr 861)

Cytoplasmic FAK (tyr 861) measurements were not significantly different in patients who died during follow-up (p=0.573, Mann-Whitney U, supplementary figure 5.75, appendix 5.3). The median cytoplasmic FAK (tyr 861) was 40.00 (IQR 3.33 – 76.67) in the alive group compared to 40.00 (IQR 6.67 – 73.33) in patients who did not survive 5 years.

The 5 year overall survival rate for patients with a raised cytoplasmic FAK (tyr 861) was 56.5% compared to 58.4% in patients with a low cytoplasmic FAK (tyr 861) expression (p=0.660, Pearson's chi square). On logrank analysis raised cytoplasmic FAK

(tyr 861) was not associated with overall survival (p=0.851). The mean survival for patients with a raised cytoplasmic FAK (tyr 861) was 46.0 months (95% CI 42.9 – 49.1) compared with 46.2 months (95% CI 44.1 – 48.3) in the low cytoplasmic FAK (tyr 861) group (supplementary figure 5.76, appendix 5.3).

On Cox univariable regression analysis raised cytoplasmic FAK (tyr 861) was not associated with overall survival when dichotomised as a categorical variable (HR 1.03 (95% CI 0.78 – 1.36), p=0.851) or when included as a continuous variable (HR 1.00 (95% CI 1.00 – 1.01), p=0.715).

Cytoplasmic FAK (tyr 861) was not predictive of death during follow-up. When ROC analysis was performed using death as the endpoint, the AUC was 0.51 (95% CI 0.47 – 0.56, p=0.574) for cytoplasmic FAK (tyr 861) as a continuous variable and an AUC of 0.50 (95% CI 0.46 – 0.55, p=0.921) for cytoplasmic FAK (tyr 861) as a categorical variable (supplementary figure 5.77, appendix 5.3).

5.4.11.9 Multivariable recurrence-free survival

Proportionality assumptions testing

All the studied variables underwent Cox proportional hazards assumption testing as described previously

Cox proportional hazards regression analysis

All covariates with a significance level of p<0.1 were included in the multivariable model. Terms were removed if their respective p-values were >0.05. On univariable analysis, advancing age (p=0.048), emergency presentation (p<0.001), advancing T-stage (p<0.001), advancing N-stage (p<0.001), higher TNM stage (p<0.001), poor differentiation (p0.002), raised serum CRP (p=0.012), hypoalbuminaemia (p=0.022) and good Klintrup score (p<0.001), raised cytoplasmic HCK (p=0.023) and raised cytoplasmic FAK (tyr861) (p=0.032) had a p-value <0.1 and were therefore included in the multivariable Cox proportional hazards regression model.

On multivariable analysis, advancing T-stage (when all subcategories were compared with T1 (T2 HR 0.57 (95% CI 0.10 – 3.10), (T3 HR 1.04 (95% CI 0.25 – 4.39) and (T4 HR 3.14 (95% CI 0.75 – 13.20)), p<0.001), advancing N-stage (when all subcategories were compared with N0 (N1 HR 2.15 (95% CI 1.40 – 3.32), (N2 HR 3.02 (95% CI 1.80 – 5.07), p<0.001), raised Klintrup score (HR 0.43 (95% CI0.25 – 0.75), p= 0.003) and raised cytoplasmic FAK (tyr 861) (HR 1.50 (95% CI 1.02 – 2.21),

p=0.040)were independently and significantly associated with poor recurrence free survival (table 5.11).

Table 5.11: The relationship between clinicopathological factors and recurrence-free survival in patients with CI colorectal cancer: univariable and multivariable analysis

	Univariable analysis			Multivariable analysis		
	Coefficient	p-value	Hazard ratio (95% CI)	Coefficient	p-value	Hazard ratio (95% CI)
Age	-0.015	0.048	0.99 (0.97 - 1.00)		0.838	
Sex						
Female			1			
Male	-0.019	0.910	0.98 (0.70 – 1.37)			
Presentation						
Elective			1			
Emergency	0.645	<0.001	1.91 (1.36 – 2.68)		0.223	
Tumour site						
Right	0.000	0.131	1			
Left	-0.397		0.67 (0.45 – 0.99)			
Rectum	-0.095		0.91 (0.60 – 1.37)			
T stage						
1	0.000	<0.001	1	0.000	<0.001	1
2	-0.186		0.83 (0.17 – 4.12)	-0.568		0.57 (0.10 – 3.10)
3	0.927		2.53 (0.62 – 10.32)	0.042		1.04 (0.25 – 4.39)
4	1.893		6.64 (1.63 – 27.11)	1.145		3.14 (0.75 – 13.20)
N stage						
0	0.000	<0.001	1	0.000	<0.001	1
1	1.048		2.85 (1.96 – 4.16)	0.791		2.15 (1.40 – 3.32)
2	1.663		5.27 (3.41 – 8.15)	1.145		3.02 (1.80 – 5.07)
TNM stage						
I	0.000	<0.001	1			
II	1.088		2.97 (1.27 – 6.92)			
III	2.090		8.09 (3.53 – 18.52)		0.499	
Differentiation						
Well/Moderate			1			
Poor	0.791	0.002	2.21 (1.34 – 3.62)		0.670	
Serum CRP						
Normal			1			
High	0.484	0.012	1.62 (1.11 – 2.36)		0.606	
Serum albumin						
Normal			1			
Low	0.466	0.022	1.59 (1.07 – 2.38)		0.232	
Klintrup score						
High			1			1
Low	-1.325	<0.001	0.27 (0.16 – 0.44)	-0.839	0.003	0.43 (0.25 – 0.75)
Cytoplasmic MMP-9						
Low			1			
High	-0.043	0.798	0.96 (0.69 – 1.33)			
Cytoplasmic FGR						
Low			1			
High	0.122	0.540	1.13 (0.77 – 1.67)			
Nuclear FGR						
Low			1			
High	0.049	0.805	1.05 (0.71 – 1.46)			
Cytoplasmic HCK						
Low			1			
High	0.414	0.023	1.51 (1.06 – 2.16)		0.599	
Cytoplasmic FAK (tyr 861)						
Low			1			1
High	0.371	0.032	1.45 (1.03 – 2.03)	-0.407	0.040	1.50 (1.02 – 2.21)

5.4.11.10 Multivariable overall survival

On univariable survival analysis, none of the SFKs or FAK (tyr861) was associated with overall survival with all p-values >0.1. Therefore, no multivariable regression model was constructed based on these biomarkers.

5.4.12 Survival, recurrence and deaths in patients with MSI colorectal cancer

During follow-up there were 10 (11.8%) recurrences and 33 (38.3%) deaths. It has been suggested that at least 10 - 25 events are required for each variable in a multivariable model. Based on the number of events noted above, no meaningful results can be generated by undertaking univariable or multivariable survival analysis exclusively on MSI patients. The relatively low number of patients and associated events makes the chance of incurring a type I or type II reporting error highly likely. Therefore, survival analysis has not been performed on the subset of patients with MSI colorectal cancer.

5.5 Cell line work

5.5.1 The effects of Src inhibitor Dasatinib on colorectal cancer cellular proliferation and apoptosis and expression of SFKs, phosphorylated Src416 and phosphorylated FAK861.

5.5.1.1 Effects of Dasatinib on HT29 and T84 proliferation and apoptosis

Effect of Dasatinib on HT29 and T84 proliferation

At 24 hours there was no apparent effect on proliferation and at 72 hours there was a uniform reduction of proliferation and widespread cell death on microscopic review, which could be due to target toxicity. Therefore, we have shown the results at 48 hours. Increasing concentrations of Dasatinib did not significantly inhibit proliferation of HT29 cells. Although there appears to be reduction in proliferation in the T84 cells, this was not in a dose dependent manner (figure 5.65).

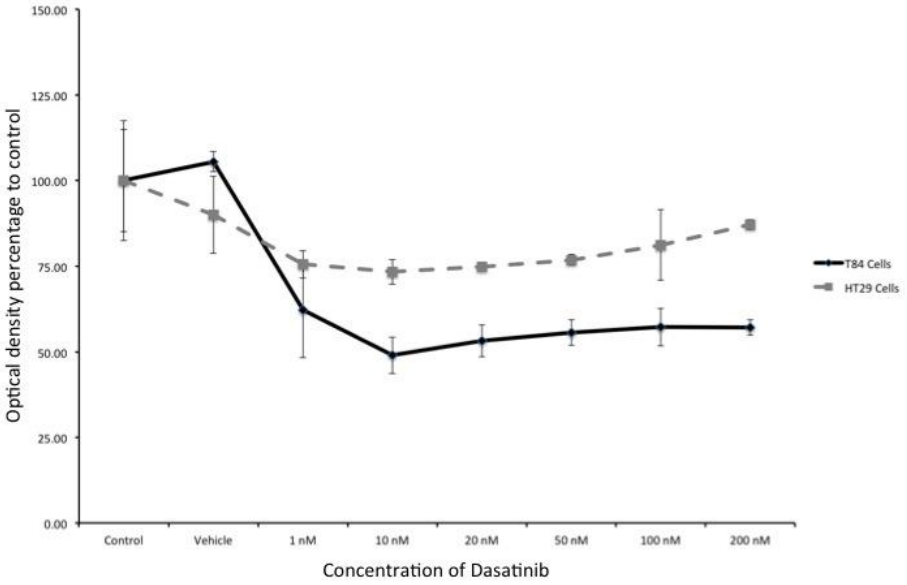


Figure 5.65: Effect of Dasatinib on HT29 and T84 proliferation

Effect of Dasatinib on cell proliferation was determined by WST-1 assay. This graph represents the results of WST-1 assay at 48 hours. There was no dose dependent reduction in proliferation. Error bars represent the standard deviation from the means of the three experiments.

Effects of Dasatinib on HT29 and T84 apoptosis

At 24 hours there was no apparent effect on apoptosis and at 72 hours there was evidence of widespread cell death on microscopic review. Therefore, as with the WST-1 experiment, we have shown the results at 48 hours. Increasing concentrations of Dasatinib did not significantly increase apoptosis of HT29 cells. However, there appears to be an increase in apoptosis of the T84 cells (figure 5.66).

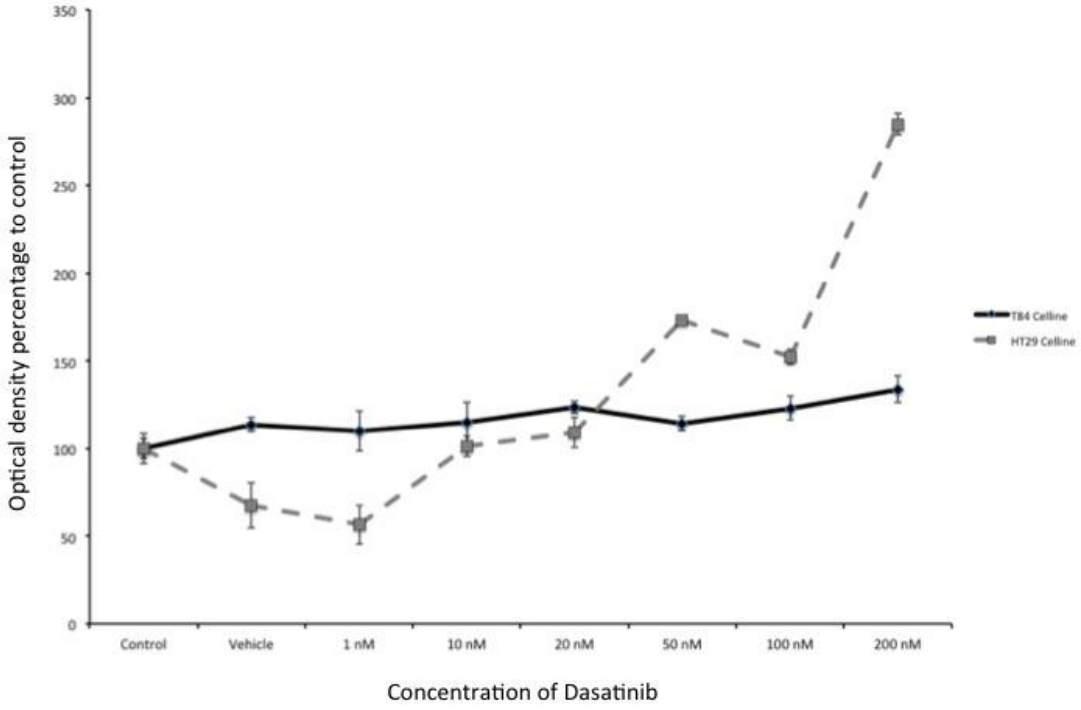


Figure 5.66: Effect of Dasatinib on HT29 and T84 apoptosis

Effect of Dasatinib on cellular apoptosis was determined by ELISA cell death detection assay. This graph represents the results of Cell Death Detection ELISA *assay at 48 hours. There was an increase in apoptosis in T84 cells but not HT29 cells. Error bars represent the standard deviation from the means of the three experiments.*

5.5.1.2 The effects of Src inhibitor Dasatinib on expression of SFKs, phosphorylated Src416 and phosphorylated FAK861 in colorectal cancer cell lines.

HT29 and T84 cell lines were used to examine the effect of Src kinase inhibitor Dasatinib on protein expression and cellular location of Src416, FAK861 and the SFKs (FGR, FYN, HCK and LCK) in cell pellets. The same antibodies employed in the previous IHC results were used in these experiments. Following the results of the proliferation and apoptosis experiments, cells were treated with 50nM of Dasatinib for 48 hours. For each study there was a control (untreated) and drug treated group. Immunoreactivity for all antibodies was scored using the weighted histoscore as described in previous sections.

Effect of Dasatinib on Src416 expression

In the untreated and treated HT29 cells, Src416 was expressed at all cellular locations. Following treatment with Dasatinib, there was a significant reduction in membrane expression (p=0.002. figure 5.67) but not cytoplasmic (p=0.132) or nuclear (p=0.937)

expression (Figure 5.67). In the untreated and treated T84 cells, Src416 was expressed at all cellular locations. Interestingly, when examining the expression levels in both treated and untreated T84 cells, neither mSrc416 (p=0.082), cSrc416 (p=0.247) or nSrc416 (p=0.177) expression showed a significant reduction in Dasatinib treated cells.

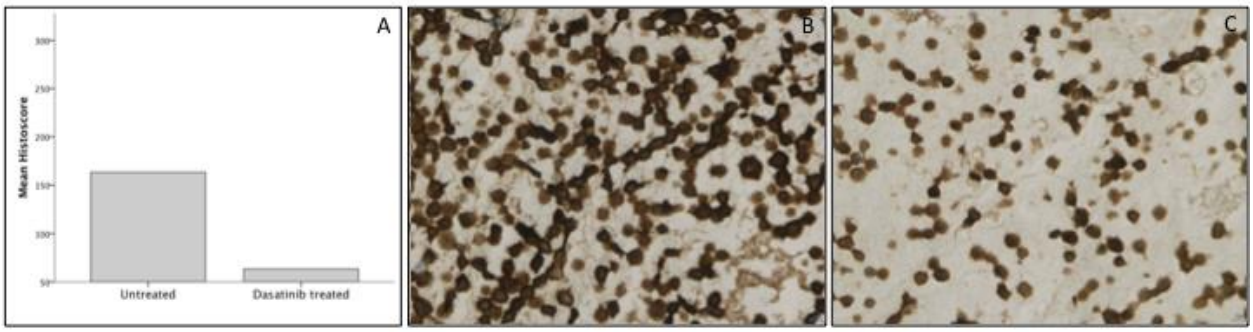


Figure 5.67: Effect of Dasatinib on mSrc416 expression in HT29 cells

A.) Bar chart demonstrating the reduction in membrane Src416 expression when comparing Dasatinib treated and untreated (media only) HT29 cells (p=0.002). B.) Demonstrates an Src416 expression in untreated HT29 cell lines. C.) Demonstrated Src416 expression in Dasatinib treated HT 29 cell lines

Effect of Dasatinib on FAK (tyr 861) expression

In the untreated and treated HT29 cells, FAK (tyr 861) was expressed at all cellular locations. Following treatment with Dasatinib, there was a significant reduction in membrane FAK (tyr 861) (p=0.004) and cytoplasmic FAK (tyr 861) (p=0.002) expression but not nuclear FAK (tyr 861) (p=0.180) expression (Figure 5.68).

In the untreated and treated T84 cells, FAK (tyr 861) was expressed at all cellular locations. Following treatment with Dasatinib, there was a significant reduction in cytoplasmic FAK (tyr 861) (p=0.016) and nuclear FAK (tyr 861) (p=0.008) expression but not membrane FAK (tyr 861) (p=0.421) expression (Figure 5.68).

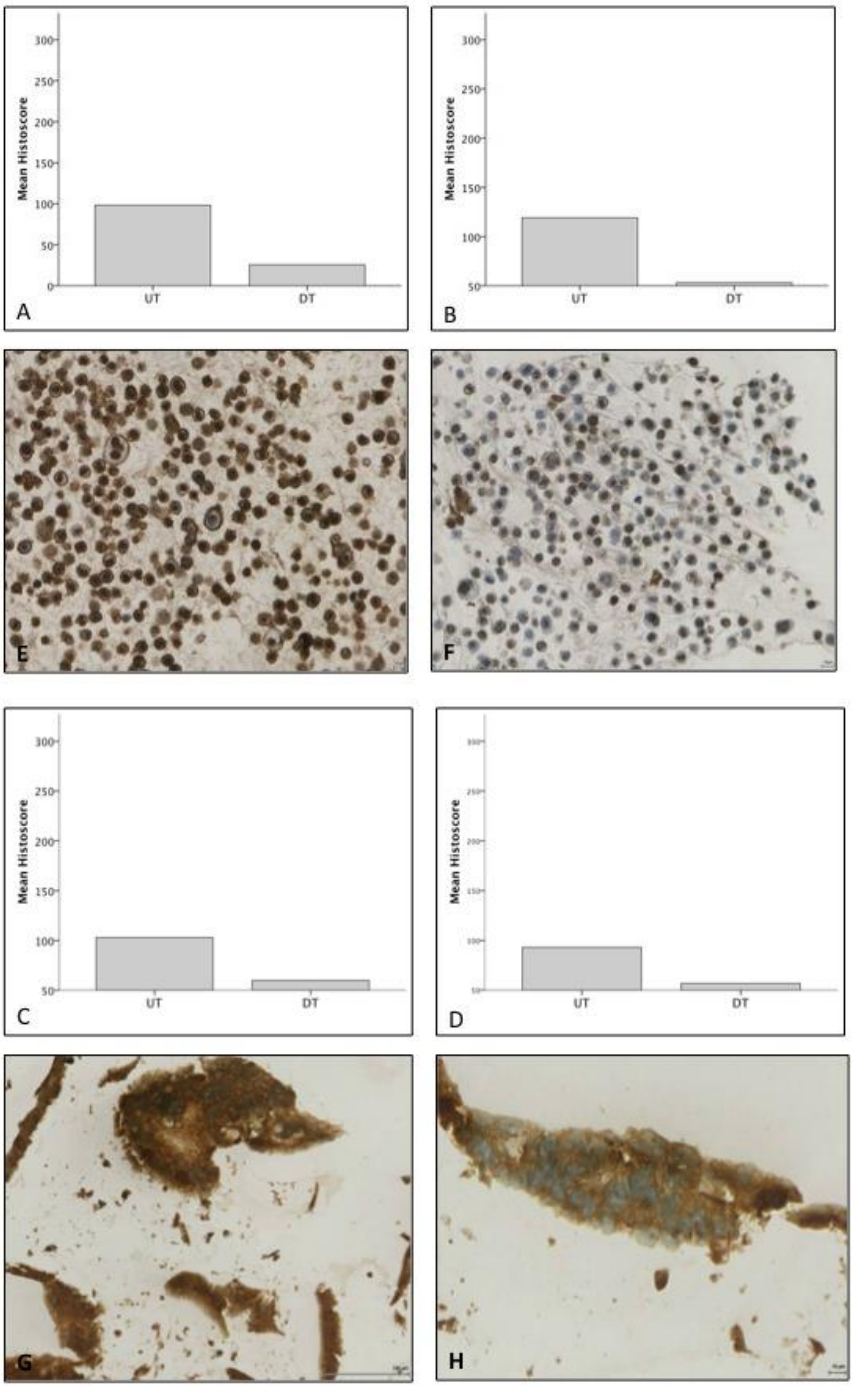


Figure 5.68: Effect of Dasatinib on FAK (tyr 861) expression in HT29 and T84 cells

A-B.) Bar chart demonstrating the reduction in membrane FAK (tyr 861) (p=0.004) and cytoplasmic FAK (tyr 861) (p=0.002) expression in HT29 cells. C-D.) Bar chart demonstrating the reduction in cytoplasmic FAK (tyr 861) (p=0.016) and nuclear FAK (tyr 861) (p=0.008) expression in T84 cells. E.) Demonstrating FAK861 expression in untreated (UT) HT29 cells. F.) Demonstrating FAK (tyr 861) expression in Dasatinib treated (DT) HT29 cells. F.) Demonstrating FAK (tyr 861) expression in untreated (UT) T84 cells. G.) Demonstrating FAK (tyr 861) expression in Dasatinib treated (DT) T84 cells.

Effect of Dasatinib on SFK expression

FGR expression

In the untreated and treated HT29 cells, FGR was expressed at all cellular locations. Following treatment with Dasatinib, there was no significant difference in membrane (p=0.065), cytoplasmic (p=0.394) or nuclear (p=0.240) expression.

In the untreated and treated T84 cells, FGR was expressed at all cellular locations. When examining the expression levels in both treated and untreated T84 cells, only nuclear FGR (p=0.004) was significantly associated with change in expression. Nuclear FGR showed an increase in expression with Dasatinib treatment. Conversely, membrane FGR showed a trend towards lower expression (p=0.065). Cytoplasmic FAK (tyr 861) showed no change in expression between controls and treated cells (p=0.310). (Figure 5.69)

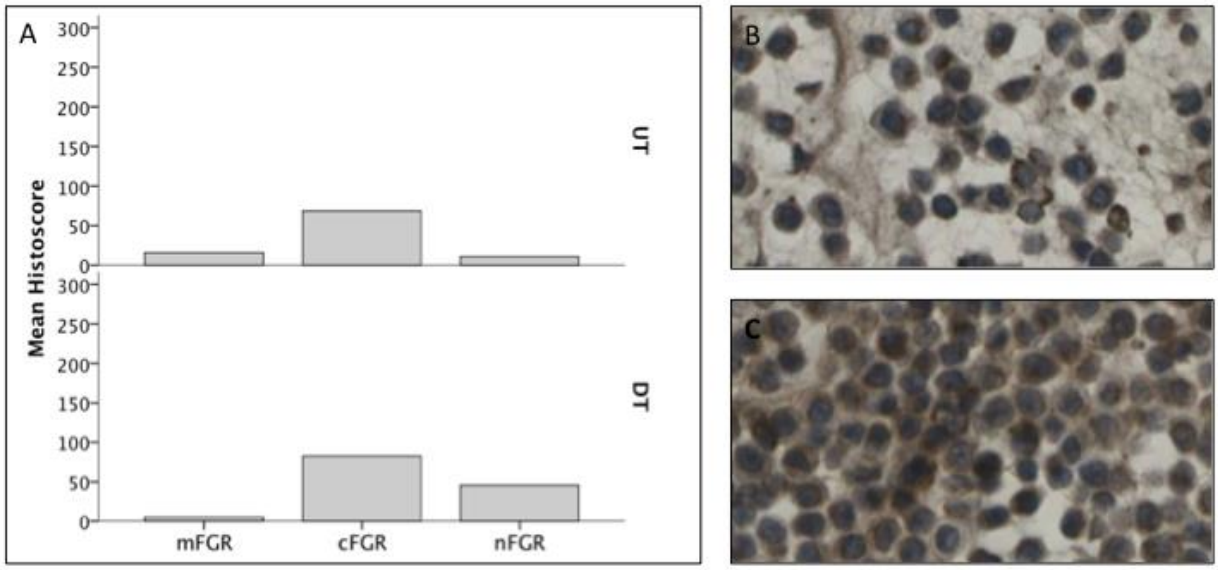


Figure 5.69: Effect of Dasatinib on cellular location of FGR expression in HT29 cells

A.) Bar chart demonstrating the trend for reduced membrane FGR expression (p=0.065) and significant increase in nuclear FGR (p=0.004) expression in Dasatinib treated cells B.) Demonstrating FGR expression in untreated (UT) HT29 cells. C.) Demonstrating FGR expression in Dasatinib treated (DT) HT29 cells.

FYN expression

In the untreated and treated HT29 cells, FYN was expressed at all cellular locations. Following treatment with Dasatinib, there was no significant difference in membrane (p=0.400), cytoplasmic (p=1.000) or nuclear (p=0.200) expression.

In the untreated and treated T84 cells, FYN was expressed at all cellular locations. Following treatment with Dasatinib, there was no significant difference in membrane (p=0.100), cytoplasmic (p=1.000) or nuclear (p=0.100) expression.

HCK expression

In the untreated and treated HT29 cells, FYN was expressed at all cellular locations. Following treatment with Dasatinib, there was no significant difference in membrane (p=0.400), cytoplasmic (p=1.000) or nuclear (p=0.200) expression.

In the untreated and treated T84 cells, FYN was expressed at all cellular locations. Following treatment with Dasatinib, there was no significant difference in membrane (p=0.100), cytoplasmic (p=1.000) or nuclear (p=0.100) expression.

LCK expression

In the untreated and treated HT29 cells, FYN was expressed at all cellular locations. Following treatment with Dasatinib, there was no significant difference in membrane (p=1.000), cytoplasmic (p=0.200) or nuclear (p=0.100) expression.

In the untreated and treated T84 cells, FYN was expressed at all cellular locations. Following treatment with Dasatinib, there was no significant difference in membrane (p=1.000), cytoplasmic (p=0.700) or nuclear (p=1.000) expression.

5.6 Discussion

5.6.1 Summary of the novel results

Allowing for the limitations in the sample size and survival model construction, the main novel finding in this chapter is that high cytoplasmic HCK expression is independently associated with poor recurrence-free survival in patients undergoing potentially curative resection for stage II colorectal cancer. Furthermore, raised cytoplasmic FAK (tyr 861) was independently associated with poor recurrence free survival in CI colorectal cancer.

5.6.2 Strengths and limitations

5.6.2.1 Strengths

This study has several strengths. Patient numbers in the validation cohort are relatively large by current standards and this offers an associated type II error probability of less than

0.1 when a 15% difference in 5 year survival rates were sought. In addition, all patients received stage-directed treatment by a specialist multidisciplinary team with considerable experience in treating colorectal cancer. The patients reside in a well-defined geographical area with minimal migration and full follow-up data is available for all patients.

In addition to the clinical factors associated with the patient outcome measures, measurements of the systemic inflammatory response namely serum CRP and albumin were performed by an accredited NHS biochemistry laboratory. The reliability of the serum CRP and albumin measurements also extends to the MSI analysis. Tumour MSI PCR analysis was performed in a tertiary referral laboratory and the reporting supervised by an expert with a special interest in Lynch syndrome diagnostics.

5.6.2.2 Limitations

The main limitations of this study are as discussed in previous chapters. These include; loss of statistical power when the cohorts were sub-divided by TNM stages and MSI status, the heterogeneity in clinicopathological factors of the sample compared to the regional population and the inability to construct a comprehensive survival model due to the absence of important clinicopathological factors such as venous invasion and BMI.

The difficulties and limitations relating to tissue dissection, tumour sampling and the accurate evaluation of biomarker expression have also been discussed previously. The non-specific binding of antibodies to non-targeted proteins during immunohistochemistry and the human error associated with scoring and is open to inaccuracies. The specific immunohistochemistry limitation of this chapter relates to the structure of the primary antibodies. The antibodies employed for identification of individual SFKs bind to areas distinct from the activation site and therefore represents both active and inactive forms of the protein and even low expressers may contain a high proportion of activated SFK. In addition, the company which manufactures the antibody which identifies the active form of the SFKs, through binding to the phosphorylated tyr416 residue, ceased production and therefore a downstream marker of Src activation (FAK (tyr 861)) was used. In reality, multiple agonists and antagonists affect the expression of FAK (tyr 861), which may not precisely represent Src activation.

Another limitation of this study is that not all family members were studied in the validation cohort. The identification of family members for evaluation in the validation cohort was based on mRNA profiling of SFKs in the frozen tissue cohort. Unfortunately, a search of online databases and published material revealed that a simultaneous quantitative

comparison of all SFKs expression has not been performed in colorectal cancer. There were studies in a renal clear cell cancer (Qayyum, 2012) and breast cancer (Elsberger, 2010) that showed C-SRC and LYN were the highest expressed members. Despite this, different family members were associated with advancing T-stage and tumour grade. Although the data presented in this thesis showed a trend in differences in expression across TNM stages, the small sample number of this cohort makes these results relatively unreliable and open to both type I and II errors. Therefore, non-studied SFKs may offer important prognostic information and a comprehensive evaluation of all SFKs is needed to be sure.

5.6.3 Discussion of the results

5.6.3.1 SFK and FAK (tyr 861) expression and survival

The influence of SFK expression on cellular behavior is relatively well understood, but, translational studies examining the prognostic role of individual SFK members are relatively few. This is the first documented report that HCK and FAK (tyr 861) are prognostic biomarkers in colorectal cancer using immunohistochemistry and confirmatory studies are needed, however, these results appear promising. In renal cancer, Lyn (Rosewier, 2016) and FAK (tyr 861) (Qayyum, 2012) were associated with poor survival and in breast cancer high c-Src expression (Elsberger, 2010) was also associated with poor outcome. These reports are from the same research group where the work for this thesis was completed and therefore independent validation of the prognostic value of SFKs in colorectal cancer is needed. Similar to the findings in this chapter, the studies referenced above have not undertaken a comprehensive evaluation of all family members when determining the prognostic value of SFKs. This makes translating a single family member into a prognostic biomarker difficult as there may be overlap with other family members and establishing real causation in this setting can be difficult.

5.6.3.2 SFK and FAK (tyr 861) expression and colorectal cancer

Allowing for the limitations in sample size, the results of the SFK mRNA profiling suggests that although Src, LYN and YES were the most highly expressed SFKs in colorectal cancer, there was no change in expression with advancing tumour stage. This observation is supported by Emaduddin *et al* who reported that SRC, LYN and YES were present in all the cell lines studied (Emaduddin, 2008). Furthermore, heterogeneity in the

expression of other SFKs were present and this observation supports the results here that FGR, FYN, HCK and LCK varied among samples and in particular with TNM stage. Given the identification of HCK as a prognostic biomarker, it is also possible that FYN and LCK are also determinents of survival.

Dasatinib activity has been studied in various cancer cell lines including colon, breast and prostate (Elsberger, 2009; Serrels, 2006; Tatarov, 2009). Here, Dasatinib was not associated with a dose dependent reduction in metabolic activity using the WST-1 assay. These findings confirm those of Serrel *et al* who observed that when Dasatinib was at a concentration sufficient to suppress SFK activation, proliferation was not inhibited in 10 out of the 12 cell lines studied (Serrel, 2006). At higher concentrations, Dasatinib was associated with a reduction in cell proliferation, which suggests that Dasatinib may inhibit proliferation independent of SFK activity, however, the mechanism has not been identified. FAK is thought to inhibit apoptosis through the PI3K/AKT pathway and caspase-3 cascade (Sonoda, 2000). These results confirm the *in vitro* evidence that FAK plays an important role in inhibiting apoptosis. Dasatinib treatment of cell lines resulted in a reduction in phosphorylated FAK (tyr 861) expression and promotion of apoptosis in the T84 cell line. These findings are clinically relevant given that loss of spontaneous apoptosis was associated with poorer response rates to neoadjuvant chemoradiotherapy in rectal cancers (Rödel, 2002).

5.6.3.3 SFK and FAK (tyr 861) as a prognostic and predictive biomarker

Cytoplasmic HCK and FAK (tyr 861) were associated with poorer recurrence free survival in the entire cohort with HCK retaining independence in TNM stage II cancers. It is unlikely, based on this evidence alone, that HCK could be used in clinical practice due to inherent limitations in the immunohistochemistry methodology. Given the homologus structure of the SFK protein, antibodies that identify activation through phosphorylation of residues in the peptide, will not discriminate between individual members. Furthermore, antibodies that identify individual members cannot discriminate between inactive and active forms of the protein and an immunohistological method for identifying an activated member has not been proposed or identified. This is likely to be important in a clinical context, as one would expect only an activated form of the protein to be biologically influential. There are situations where a biomarker requires two techniques for pathological reporting such as the use of Her2 in breast cancer. It is possible that reporting of an SFK may require IHC for the both individual member and a biomarker for SFK

activation such as phosphorylation of tyr416 or quantifying the expression of FAK (tyr 861). Using FAK (tyr 861) for this purpose is limited given the likely multiple sources for agonism and antagonism that influence the expression and activation of the protein. Methodologies that use whole tumour homogenate such as PCR or Western blots are unlikely to be useful as the subcellular location of the protein is an important determinant of cellular behavior. If SFKs and markers of their activation are to be employed as prognostic or predictive biomarkers, a robust and reliable methodology for identification and quantification is needed, irrespective of how challenging this is likely to be.
Despite growing evidence that SFKs play an important role in cancer progression and metastasis, clinical trials of Src inhibitors have been disappointing (Sharma, 2012). Similar to HER2 positivity and Herceptin, it is possible that patients with a particularly high expression and activation of one of the family members may have a beneficial response to Src inhibitor therapy. Treating all patients with Src inhibitor therapy is unlikely to be biologically or financially appropriate. Retrospective analysis of clinical trials by correlating response to treatment with expression of SFKs and or markers of their activation will possibly add weight for an adequately powered prospective study of Src inhibitor therapy and its predictive biomarker in identifying patients likely to benefit from Src inhibitor therapies. It appears that a significant barrier to implementing biomarkers into clinical practice is reliable methodology for biomarker identification, quantification, reporting and availability of a specific therapy with a favorable benefit/side effect profile. Until these issues have been addressed, it is unlikely that Src inhibitor therapy can be incorporated into the management process of colorectal cancer.

5.6.3.4 Introducing SFK and FAK (tyr 861) expression into the prognostication process in colorectal cancer

Following potentially curative resection, patients are offered adjuvant chemotherapy based on their expected risk of recurrence. High risk features currently include T4 disease, lymph node metastasis and vascular invasion. As discussed previously, the TNM staging system is the main biomarker for outcome prognostication; however, its predictive value could be improved. Therefore, methods to strengthen the predictive value of the TNM system with supplementary biomarkers may help improve the stratification of patients to adjuvant treatment on a need/benefit basis.

There is a clear need for treatment stratifying biomarkers in colorectal cancer, however, it remains unclear how expression of SFKs and their activation in particular

cytoplasmic HCK and FAK (tyr 861) could be integrated with TNM staging. The distinction between staging, prognosis and predictive biomarkers is important given the influence of both on survival and treatment. The limitations surrounding the predictive value of the TNM stage have been discussed previously. The main changes to managment strategies in other cancers relate to predicting response to novel therapies such as that seen in the management of breast cancer. Due to the limitations of the study, how cytoplasmic HCK or FAK (tyr 861) might fit into a staging system that includes MSI, the inflammatory responses and MMP-9 reamains unclear and cannot be answered by the results of this study. Despite these limitations, the survival model for TNM stage II colorectal cancer revealed that cytoplasmic HCK was independent of T-stage with loss of statistical significance for MSI and systemic inflammation and this is a promising avenue for further study.

5.6.4 Future direction

1. A comprehensive study of all SFKs and FAK (tyr 861) needs to be conducted in an adequately powered independent cohort with sufficient patient numbers and events so that survival modeling can be performed using the sub classification methods in this chapter.

2. Using gene silencing and Src inhibitor therapy to identify the role that each SFK plays in colorectal cancer cellular behavior. This could support the use of SFKs as predictive biomarkers for Src inhibitor therapy

3. Correlate the expression of SFKs with response to Src inhibitor treatment in patients entered in clinical trials for colorectal cancer. If successful in demonstrating that SFKs predict response to Src inhibitor therapy, this treatment regimen could be translated into high risk patients who have undergone potentially curative resection.

5.6.5 Conclusion

Src family kinases are a promising therapeutic target for patients at high risk of recurrence. Allowing for the limitations in the data sample, cytoplasmic HCK offers additional prognostic information beyond that seen with commonly used clinicopathological factors in patients with stage II colorectal cancer. Cancer cell responses to Src inhibitor therapy, as measured by metabolic activity and apoptosis, varies between individual cell lines and

reflects the tumours molecular heterogeneity. SFKs may offer predictive as well as prognostic information that will help identify patients for Src inhibitor therapy and this requires further study.

6. General Discussion

6.1 Summary of the novel findings in this thesis

The main novel findings of the studies reported in this thesis are threefold:
1. That MSI tumours are associated with a systemic inflammatory response indicate by the measurement of acute phase proteins serum CRP and albumin.

2. Serum MMP-9 is associated with tumoural MMP-9 expression and poor survival in a pilot study of 95 patients undergoing potentially curative resection for TNM stage I-III colorectal cancer.

3. Cytoplasmic HCK is independently associated with poor recurrence-free survival in patients undergoing potentially curative resection for TNM stage II colorectal cancer.

6.2 Strengths and limitations

6.2.1 Strengths

This study has several strengths. Patient numbers in the validation cohort are relatively large by current standards and this offers an associated type II error probability of less than 0.1 when a 15% difference in 5 year survival rates were sought. In addition, all patients received stage-directed treatment by a specialist multidisciplinary team with considerable experience in treating colorectal cancer. The patients reside in a well-defined geographical area with minimal migration and full-follow up data is available for all patients.

In addition to the clinical factors associated with the patient outcome measures, measurements of the systemic inflammatory response namely serum CRP and albumin were performed by an accredited NHS biochemistry laboratory. The reliability of the serum CRP and albumin measurements also extends to the MSI analysis. Tumour MSI PCR analysis was performed in a tertiary referral laboratory and the reporting supervised by an expert with a special interest in Lynch syndrome diagnostics.

6.2.2 Limitations

6.2.2.1 Data sampling and power

The main limitation of this study is the statistical power available when the cohorts were sub-stratified by TNM stages and MSI status. The MSI and TNM stage I groups had small sample sizes, and too few events for reliable survival analysis, which was not performed. The best method for determining adequate power in survival analysis is controversial, but, two methods have been proposed. Firstly, power the sample for an expected difference in survival rates across a fixed time frame and secondly, powering for an anticipated number of survival events in a particular group of patients. Both methods have strengths and weaknesses and both were employed in this thesis. Although there was a lack of power when patients were stratified into TNM sub-groups, there were sufficient events available for multivariable modeling.

Another limitation of this study is the quality of the patient sample. The frequencies of the core clinicopathological factors for the patient cohorts as well as the regional data for the study time frame have been included in the results chapters. The clinicopathological frequencies in the pilot study were different to those observed in the validation cohort and the regional population. This would normally mean interpreting the pilot results with caution, however, including a validation cohort with no overlapping of patients adds some reliability to the data findings. The main difference between the cohorts was the proportion of emergency patients in the training cohort (cohort 1). The reason for this difference is not clear and may reflect the way patients were identified for inclusion in the study. Patients for the validation cohort (cohort 2) were identified by searching a pathology database for all colorectal cancer specimens over a particular period, whilst patients for the training cohort were identified from a prospectively maintained database of colorectal resections.

A relative limitation of this study is the strength of the chosen survival endpoints. Death is a relatively robust endpoint, however, the cause of death is dependent upon the interpretation of the clinical situation by the clinician writing the death certificate. This limitation also applies to the process of determining if cancer recurrence has developed or not. Furthermore, patients who die of an unrelated illness may harbor cancer recurrence. Although CT scans can detect cancer recurrence at a very early stage, the presence of a normal scan does not rule out the presence of micro-metastasis. Therefore, determining the exact timing of cancer recurrence is challenging.

6.2.2.2 Pathological factors

A relative limitation of this study is the absence of some previously reported prognostic clinicopathological factors in the multivariable model. Although core factors such as TNM stage and its components were included there were omissions such as BMI and vascular invasion. BMI is not included in the core data set for MDT discussion because of the associated limitations in its recording and time dependency. It has been reported that detection of venous invasion can be increased to 18% from 56% by using elastic H&E staining (Roxburgh, 2010). This method of detection is not currently universally employed and only a small proportion of patients in cohorts 1 and 2 underwent elastic H&E assessment. Therefore, with H&E assessment alone being associated with a 38% false negative rate, vascular invasion was not included in the multivariable model. BMI is a modifiable clinicopathological factors that can be considered as time dependent. Changes in living circumstances may alter a patients' BMI and thus the biological effect of this covariate on the pathophysiology of the host and the cancer varies with time. This may explain why the effect of these factors with prognosis remains poorly understood with results of a metanalysis offering conflicting patterns of results (Lee, 2015).

A relative limitation of this study is that tumours were dissected macroscopically rather than microscopically to increase the percentage of tumour DNA in the sample. It has been estimated that a sample requires at least 40% tumour DNA to detect the microsatellite repeats on PCR. Factors that potentially dilute the tumour DNA include lower T stage tumours and the presence of a pronounced inflammatory infiltrate. These features increase the volume of non-cancerous DNA thus increasing the false negative detection rate. MSI tumours, which are associated with a pronounced inflammatory infiltrate and lower T-stage tumours, which have a higher proportion of adjacent adenoma, may require dissection. Macroscopic dissection may inadvertently include a proportion of these non-cancerous cells in the sample thus diluting the tumour DNA yield. To control for this, all tumours underwent MMR protein assessment through immunohistochemistry.

The non-specific binding of antibodies to non-targeted proteins during immunohistochemistry and the non-automated quantification method is associated with a risk of human error and is not entirely accurate. The specific immunohistochemistry limitations of this thesis relates to the structure of the primary antibodies. The antibodies employed for identification of proteins bind to an area distinct from the activation site and therefore represents both active and inactive forms of the protein and even low expressers may contain a high proportion of activated protein. Furthermore, the company that manusfactured the

antibody which identifies the active form of SFKs ceased production and therefore a downstream markers of Src activation (FAK (tyr 861)) was used.

Another limitation of this study is that not all family members were studied in the validation cohort. The identification of family members for evaluation in the validation cohort was based on mRNA profiling of SFKs in the frozen tissue cohort. Although trends in differences in expression were observed across TNM stages, the small sample number of this cohort makes these results relatively unreliable and open to type I and II errors. Therefore, omitted SFKs may offer important prognostic information and a comprehensive evaluation of all SFKs is likely needed.

6.3 Discussion of the results

Taken into consideration the results of previous chapters and supported by evidence from the literature, there appears to be an interaction between the host and the tumour. This is particularly important when considering the interaction between systemic inflammation and microsatellite instability in colorectal cancer. Evidence from patients with metabolic syndrome and increased global methylation suggest a possible cause of MSI colorectal cancer through hypermethylation and subsequent silencing of the MLH1 protein. Despite this observation patients with MSI cancer have a relatively improved survival. This likely relates to the pronounced lymphocytic infiltrate associated with the cancers that possibly negates the deleterious effect of the systemic inflammatory response. How the host interacts with molecular behaviors of the cancer remains unknown. The cardinal features associated with every cancer, cell proliferation, loss of apoptosis, loss of cell adhesion and migration to name but a few are ultimately controlled by signaling pathways which receive external stimuli from the cell membrane. Cell line studies supported by mouse models show that IL6, an important pro-inflammatory cytokine, stimulates proliferation and inhibits apoptosis through activation of STAT3. This ultimately results in larger cancers, which is also a feature of the MSI pathway. It remains unclear what the exact aetiological factor for systemic inflammation in cancer is. Possible causes include patient related factors such as poor health and aggressive tumours with resultant complications such as perforation or obstruction. Unfortunately given the limitations of the data contained in this thesis the relationship between systemic inflammation, MSI, MMP9 and expression and activation of SFK could not be delineated.

Another feature of this thesis is the heterogeneity of the prognostic value of biomarkers relating to the cancer related tissue of origin. Specifically, MMP9 in the serum was significantly associated with poorer survival however this was not confirmed in the

tumour which may represent a type II reporting error. This raises the possibility that studying the tumour alone is not always the best way of undertaking disease prognostication. The majority of biomarkers available in clinical practice are related to the expression of the molecule within the tumour cell. The results contained within this thesis suggest that sampling of blood, especially given the identification of circulating tumour cells, may be a better method. The finding that MMP-9 in the serum was also associated with advanced tumour stage and tumour expression further supports the notion of an interaction between tumour and host. MMP-9 degrades connective tissue, allowing the passage of cells through the stroma. This is advantageous for immune cells but is clearly an important factor for metastases for colorectal cancer cells. Unfortunately trials of MMP-9 inhibitors have not reported promising results and although this is disappointing it does not rule out MMP-9 as a predictive biomarker for identifying patients for treatment. In addition to the lack of response from the tumour there is no highly selective MMP-9 inhibitor available and therefore currently used compounds inhibit all types of MMPs. The influence this has on the tumour remains unknown and possibly has both anti-tumour and pro-tumour effects and this requires further pre-clinical studies. The identification of MMP-9 in the serum has far reaching effects on other biomarkers. The identification of circulating tumour cells or DNA offers a promising avenue for interrogating the molecular biology of the tumour without having to undergo invasive sampling techniques. Indeed, these cells may reflect the most aggressive behaviors of the cancer and may offer superior prognostic information and this remains unknown requiring further study. Taking into consideration all these finding s the emergence of newer biomarkers is likely to require a robust scientific methodology coupled with the best forms of tissue sampling. This will hopefully result in an improved personalized prediction model that will translate into improved survival rates.

Currently the best available stratification model for planning treatment is the TNM staging system. Although this relatively simple model has good prognostic power its prediction for treatment stratification needs improved. Particularly in patients with TNM stage II disease who have an approximately 30% chance of recurrence. Following curative resection patients who have either stage 3 disease, T4 tumours or vascular invasion are offered adjuvant chemotherapy. Despite this, a proportion of patients who have undergone chemotherapy as well as some patients regarded, as low risk will develop disease recurrence. Therefore it is likely that not all patients who receive adjuvant chemotherapy benefit and this is supported by the observations of Roedel *et al* who observed that only 20% of patients have a complete pathological response to neoadjuvant chemotherapy for rectal cancer (Roedel, 2010). Furthermore, patients with MSI colorectal cancer are relatively chemo resistant to 5

FU based chemotherapy regimens. There is a clinical need to identify patients who need and will benefit from adjuvant treatments. The model of biomarker driven treatments has been particularly useful in breast cancer where patients with tumours expressing ER are given ER antagonists. It is possible that a similar model would work for colorectal cancer, given a suitable target. Unfortunately the limitations of the data within this thesis do not entirely support the integration of systemic inflammation, MMP-9 and expression or activation of SFK into current treatment planning. The observation that cytoplasmic HCK was an independent factor for prognosis in TNM stage 2 colorectal cancer is promising and it may offer a novel therapeutic target. Dasatinib, a SRK inhibitor, promotes cellular apoptosis in metastatic colorectal cancer cell lines, which has also been observed by others. Combined with a robust methodology for identifying and quantifying SFK related biomarkers may help incorporate this into clinical practice.

Although there are promising results in this thesis it also highlights the substantial challenges associated with prognostic studies. The vast majority of biomarker studies in the literature are underpowered with methodological variations in which may explain why despite apparently compelling pre-clinical evidence, so few biomarkers make it into clinical practice. The introduction of the REMARK criteria for biomarker reporting may go some way to improving the quality of biomarker reporting. Ultimately the factors that influence patient survival are numerous and reflect both host and tumour behavior. This makes accurate survival modeling difficult as few studies exhaustively incorporate all relevant prognostic variables. The main hurdle for translating biomarker research into clinical application relates to the coupling of a scientifically robust methodology for biomarker quantification and a drug with a favorable side effect and cost profile. Future work needs to address these issues if personalized treatments are to become integrated into clinical practice.

6.4 Future direction

1. Given the lack of power associated with studying survival associations in each TNM stage and MSI tumours, future work will need to validate these findings in a large sample size, which is adequately powered.

2. How the SIR influences the development of MSI colorectal cancer is unclear, but there is a possible link with DNA methylation. Future work examining the association between the SIR, DNA methylation, CIMP and MSI colorectal cancer which is supported by mechanistic experiments could help identify therapeutic targets for MSI colorectal cancer prevention.

3. The biological reason for the association between the SIR and survival remains unknown. Future work examining the relationship between the SIR and cell signaling pathways such as JAK/STAT3 may help identify novel therapeutic targets.

4. The results of examining the relationships between serum MMP-9 and survival in a larger cohort with tissue analysis using full tissue sections for cancer and stroma MMP-9 expression quantification could support the use of MMP-9 in future staging methodologies.

5. Looking at the effect of MMP-9 inhibitors on tumour MMP-9 expression and what happens to tumour behavior

6. Trial of MMP-9 inhibitors in patients with high expression of serum and/or tumour MMP-9

7. A comprehensive study of all SFKs and FAK (tyr 861) needs to be conducted in an adequately powered independent cohort with sufficient patient numbers and events so that survival modeling can be performed using the sub classification methods in this chapter.

8. Using gene silencing and Src inhibitor therapy identify the role that each SFK plays in colorectal cancer cellular behavior. This will support the use of a SFK as a predictive biomarker for Src inhibitor therapy

9. Correlate the expression of SFKs with response to Src inhibitor treatment in patients entered in clinical trials for colorectal cancer. If successful in demonstrating that SFKs predict response to Src inhibitor therapy, this treatment regimen could be translated into high risk patients who have undergone potentially curative resection.

6.5 Conclusion

The results of this thesis confirm that colorectal cancer is a complex disease that represents several subtypes of cancer based on molecular biological behaviors. This thesis concentrated on features of the disease related to inflammation in terms of genetic and molecular characterisation. MSI cancers are closely associated with systemic inflammation but despite this observation, they retain their relatively improved survival. MMP-9 is a feature of tissue remodeling during inflammation and is also associated with degradation of connective tissue,

advanced T-stage and poor outcome when measured in the serum. The lack of stromal quantification due to TMA use rather than full sections makes the value of tumoural MMP-9 immunoreactivity in the prognostication and its association with MSI unknown and requires further study. Finally, Src family kinase activation was also associated with systemic inflammation, however, only cytoplasmic HCK was independently associated with poor survival in patients with TNM stage II disease, the group of patients where identifying a novel biomarker is most needed. There is still some way to go before these biomarkers are translated into clinical practice and future work needs to focus on obtaining a reliable and robust scientific technique with validation in an adequately powered independent cohort.

7. Appendix

Appendix 1 – Maps of the training and validation cohort TMA's

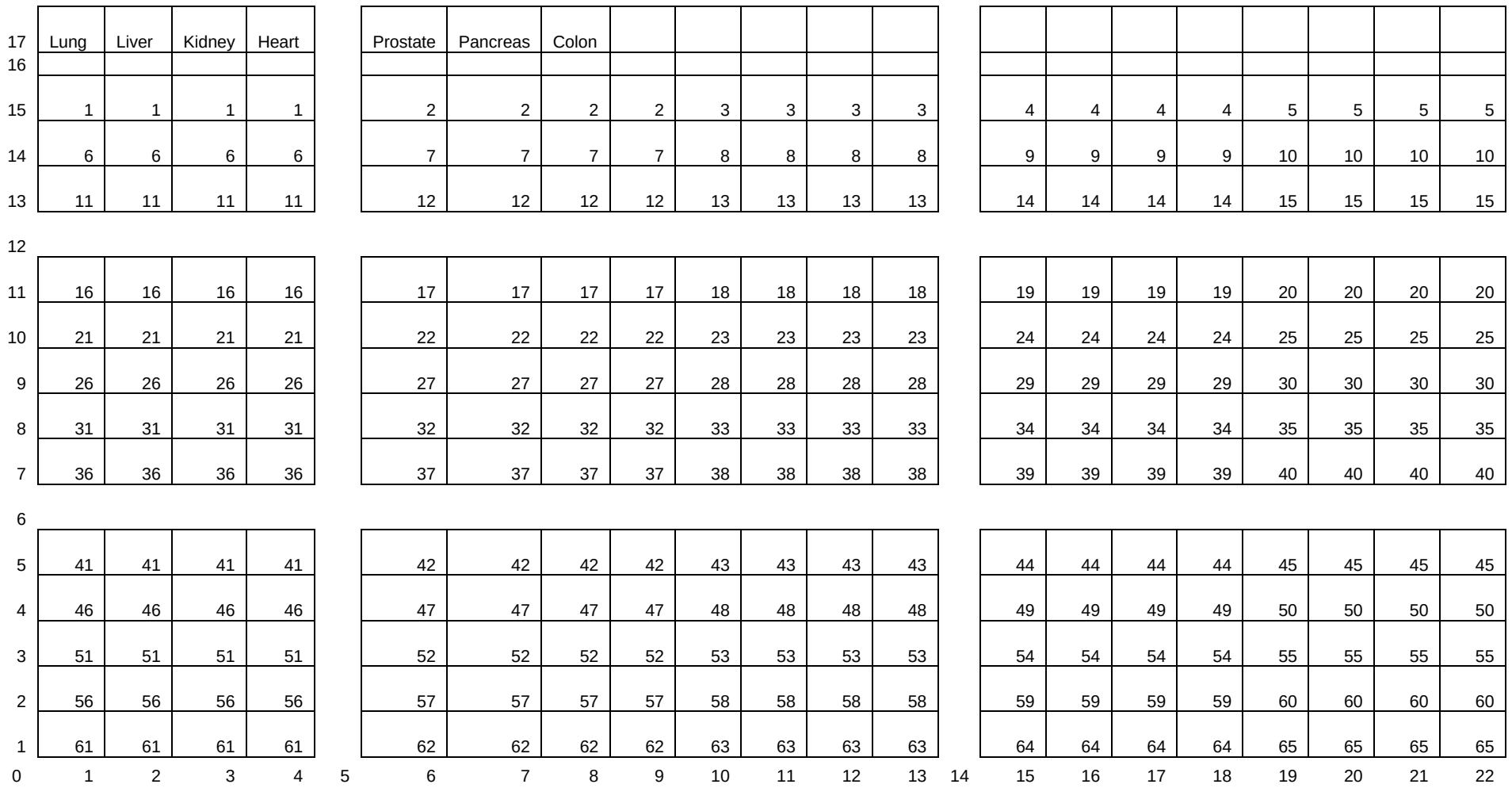
Appendix 2 – The distribution of continuous clinical data measurements

Appendix 3 – Studied variables' 5 year disease free and overall survival rates

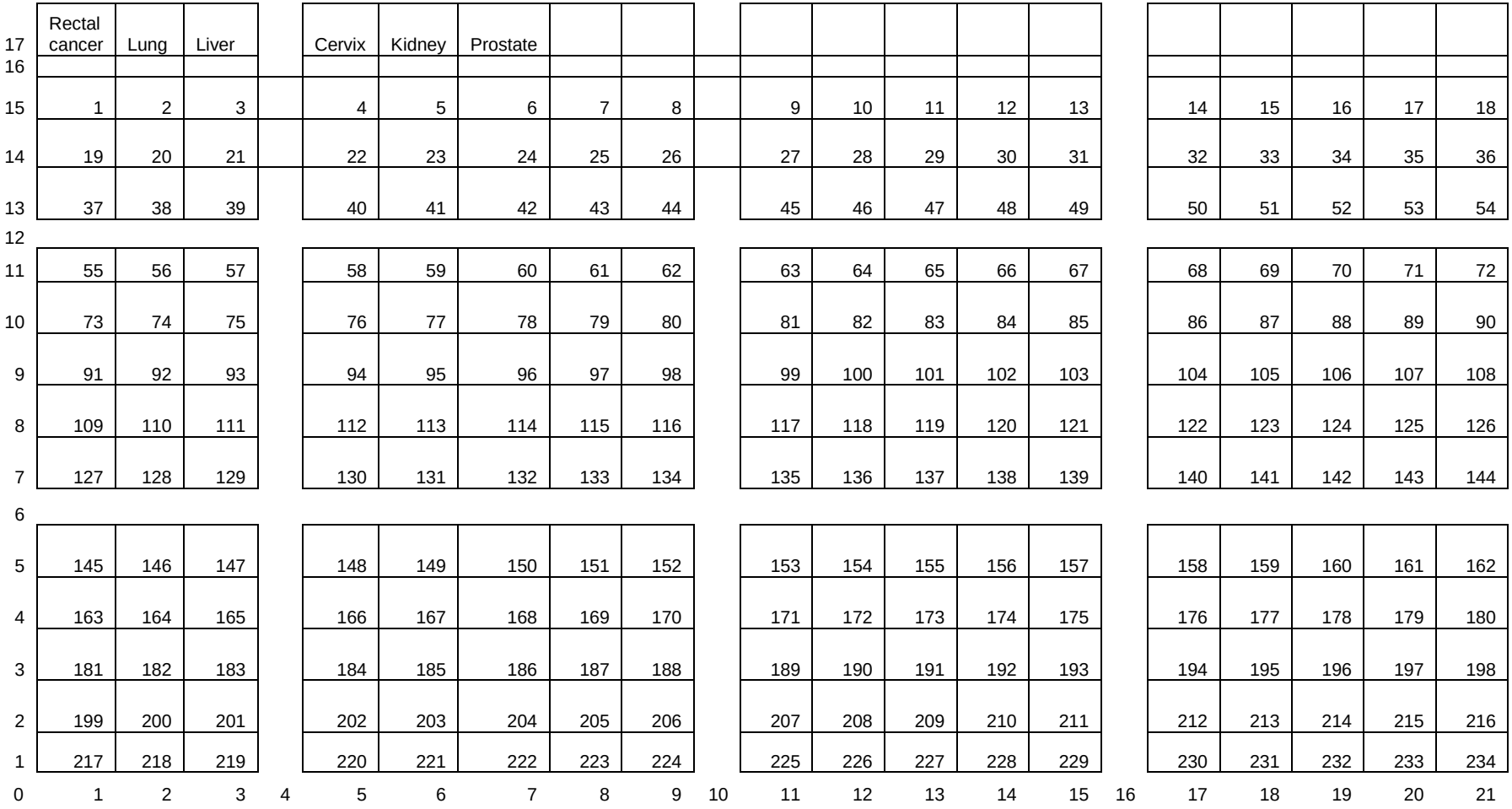
Appendix 4 – Biomarker quantification reproducibility

Appendix 5 – Supplementary data from the results chapters

Appendix 1.1 Cohort 1 TMA



Appendix 1.2 Cohort 2 TMA

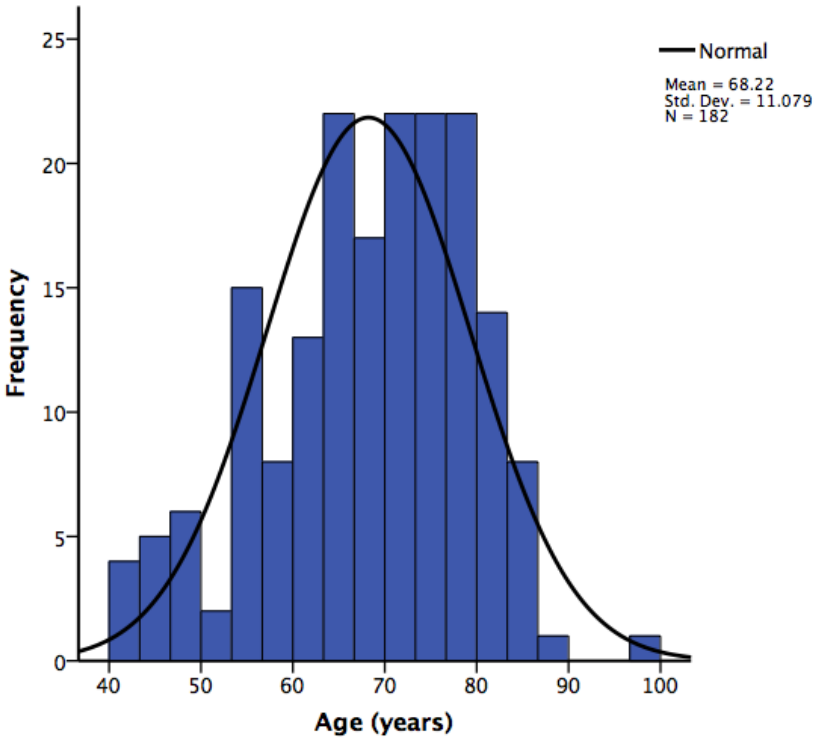


Appendix 2 – The distribution of continuous clinical data measurements

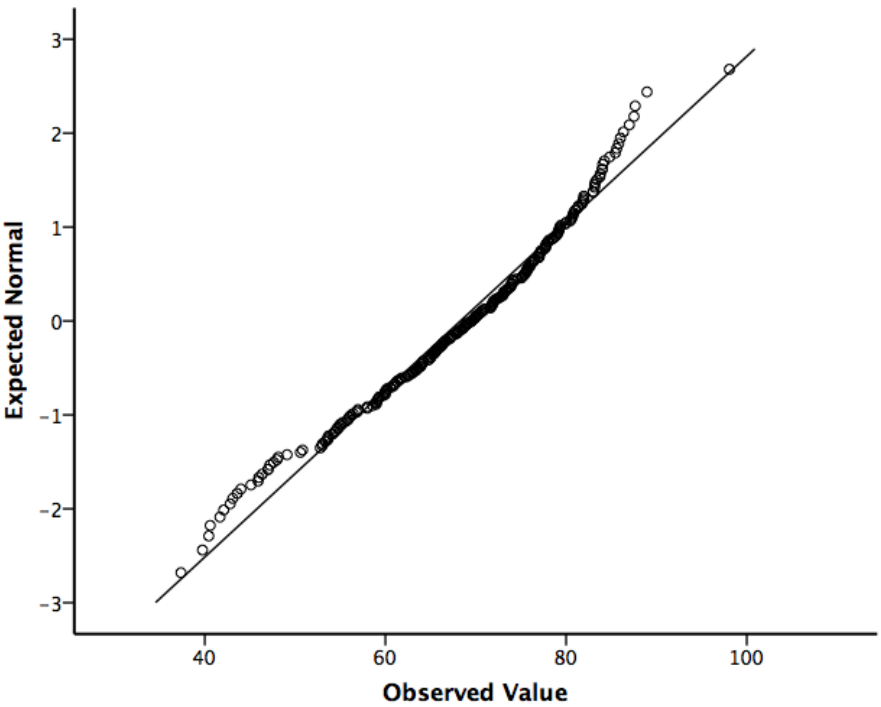
Training cohort

1. Age

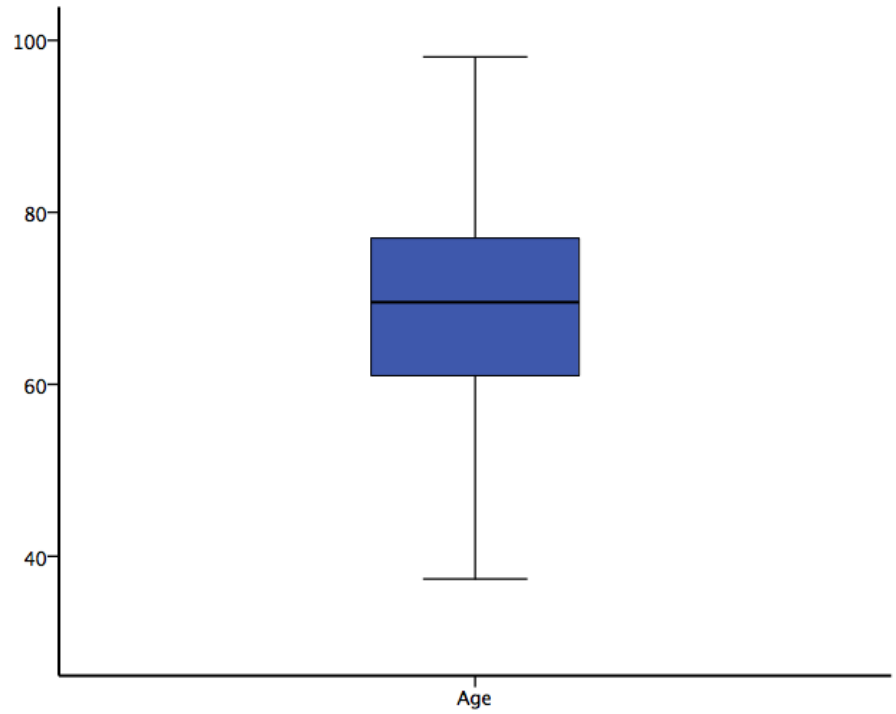
Histogram



Normal Q-Q Plot



Boxplot



Shapiro-Wilk Test

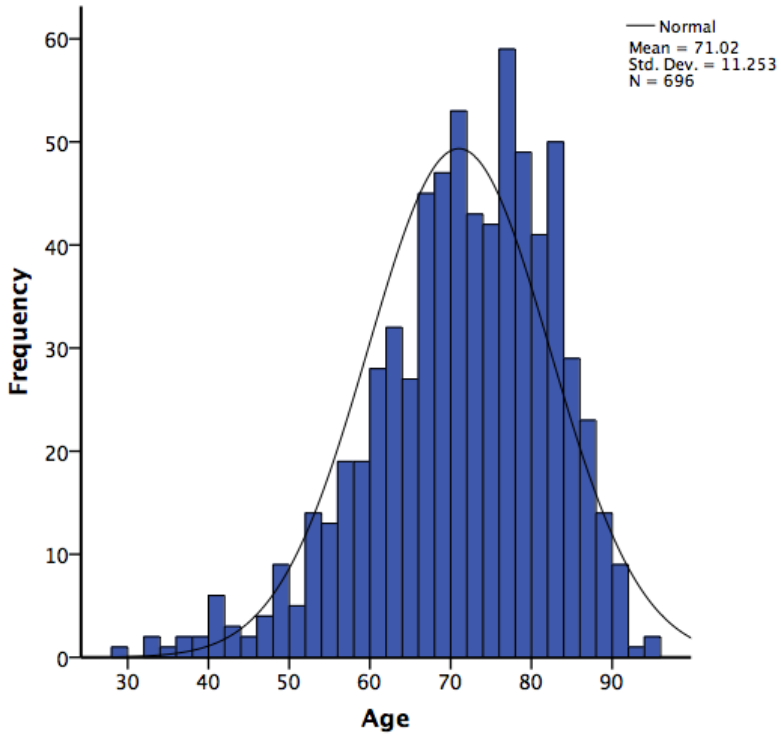
Statistic = 0.974

Degrees of freedom = 182

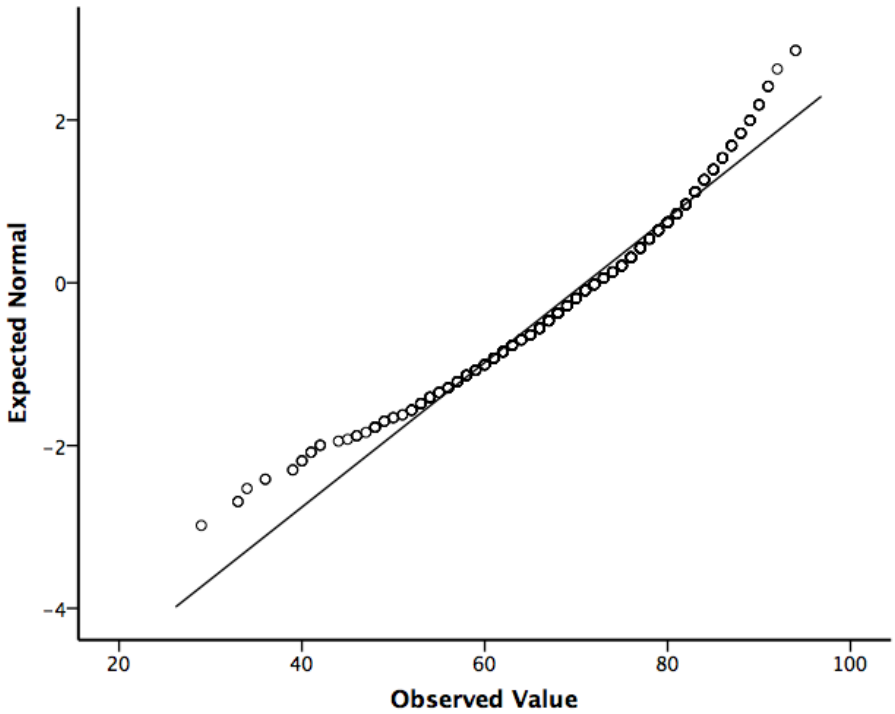
Significance = 0.002

Validation cohort

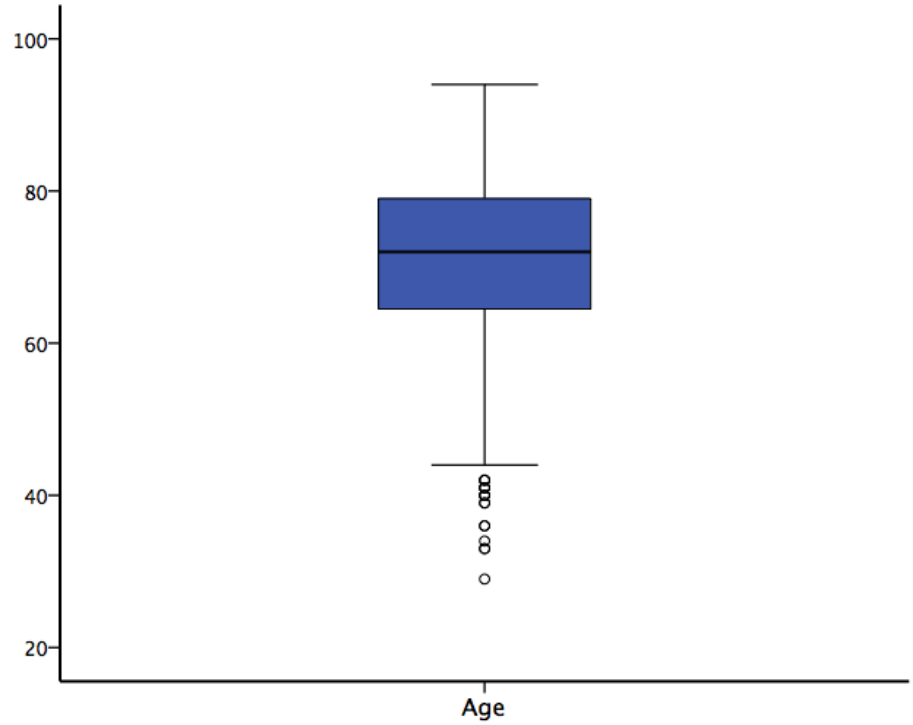
Histogram



Normal Q-Q Plot



Boxplot



Shapiro-Wilk Test

Statistic = 0.967

Degrees of freedom = 696

Significance = <0.001

Appendix 3.1: Studied variables' 5 year recurrence-free and overall survival rates (n=182) – Training cohort (cohort 1)

Biomarkers	5 Year recurrence-free survival	5 Year overall survival
Sex		
Female	55.8%	61.0%
Male	56.2%	64.8%
Presentation		
Elective	56.3%	63.8%
Emergency	50.0%	50.0%
Tumour Site		
Colon	56.1%	64.2%
Rectum	55.9%	61.0%
Tumour site enhanced		
Right	56.5%	62.3%
Left	55.6%	66.7%
Rectum	55.9%	61.0%
Differentiation		
Well-Mod	57.4%	64.8%
Poor	45.0%	50.0%
T stage		
1	100.0%	100.0%
2	69.2%	76.9%
3	59.5%	66.7%
4	42.6%	50.0%
N stage		
0	67.3%	72.4%
1	44.8%	56.7%
2	35.3%	35.3%
TNM stage (simplified)		
I	75.0%	83.3%
II	66.3%	70.9%
III	42.9%	52.4%
Serum CRP		
Normal	68.0%	74.0%
High	41.5%	50.0%
Serum albumin		
Normal	58.6%	65.6%
Low	40.0%	48.0%
Klintrup score		
Good	70.2%	77.2%
Poor	50.0%	57.3%
Tumour MMR expression	53.6%	60.8%
Proficient	69.0%	75.9%
Deficient		

Appendix 3.2: Studied variables' 5 year recurrence-free and overall survival rates (n=677) – Validation cohort (cohort 2)

Biomarkers	5 Year recurrence-free survival	5 Year overall survival
Sex		
Female	54.2%	58.4%
Male	52.5%	56.5%
Presentation		
Elective	60.5%	63.9%
Emergency	36.3%	42.3%
Tumour Site		
Colon	52.5%	56.4%
Rectum	55.2%	61.5%
Tumour site enhanced		
Right	50.0%	53.6%
Left	57.1%	60.5%
Rectum	54.2%	60.6%
Differentiation		
Well-Mod	55.2%	59.4%
Poor	36.4%	39.4%
T stage		
1	74.1%	74.1%
2	71.4%	75.8%
3	56.8%	60.1%
4	35.9%	41.9%
N stage		
0	61.7%	64.4%
1	41.6%	50.6%
2	29.2%	31.9%
TNM stage (simplified)		
I	72.1%	75.0%
II	58.5%	61.0%
III	38.4%	45.3%
Serum CRP		
Normal	64.8%	70.7%
High	43.0%	45.2%
Serum albumin		
Normal	61.7%	66.1%
Low	34.0%	38.0%
Klintrup score		
Good	66.5%	71.4%
Poor	47.6%	51.4%
MSI status		
Stable	53.5%	57.8%
Unstable	51.8%	55.3%
Cytoplasmic MMP-9		
Low	54.8%	59.0%
High	51.8%	55.7%
Cytoplasmic FGR		
Low	53.0%	57.1%
High	54.5%	58.6%

Nuclear FGR		
Low	52.7%	57.9%
High	55.3%	55.9%
Cytoplasmic HCK		
Low	54.6%	58.5%
High	49.4%	54.3%
Cytoplasmic FAK (tyr 861)		
Low	54.2%	58.5%
High	51.4%	55.2%

Appendix 3.3: Studied variables' 5 year recurrence-free and overall survival rates in patients with TNM stage II disease (n=327) – Validation cohort (cohort 2)

Biomarkers	5 Year recurrence-free survival	5 Year overall survival
Sex		
Female	60.8%	63.9%
Male	56.2%	58.0%
Presentation		
Elective	64.3%	66.1%
Emergency	46.2%	50.0%
Tumour site enhanced		
Right	58.5%	59.9%
Left	56.4%	59.8%
Rectum	61.9%	65.1%
Differentiation		
Well-Mod	59.2%	61.9%
Poor	50.0%	50.0%
T stage		
1	N/A	N/A
2	N/A	N/A
3	63.6%	66.2%
4	47.1%	49.0%
Serum CRP		
Normal	67.2%	72.1%
High	49.6%	51.1%
Serum albumin		
Normal	66.0%	69.1%
Low	39.7%	42.3%
Klintrup score		
Good	56.2%	57.9%
Poor	63.8%	68.1%
MSI status		
Stable	58.1%	61.0%
Unstable	60.0%	60.0%
Cytoplasmic MMP-9		
Low	60.0%	61.4%
High	56.8%	60.4%
Cytoplasmic FGR		
Low	56.7%	58.8%
High	63.4%	67.1%
Nuclear FGR		
Low	59.5%	62.6%
High	54.3%	54.3%
Cytoplasmic HCK		
Low	59.5%	62.3%
High	55.0%	56.2%
Cytoplasmic FAK (tyr 861)		
Low	59.5%	61.8%
High	56.1%	58.9%

Appendix 3.4: Studied variables' 5 year recurrence-free and overall survival rates in patients with TNM stage III disease (n=245) – Validation cohort (cohort 2)

Biomarkers	5 Year recurrence-free survival	5 Year overall survival
Sex		
Female	38.7%	45.2%
Male	38.0%	45.5%
Presentation		
Elective	46.8%	52.2%
Emergency	23.0%	32.2%
Tumour site enhanced		
Right	33.1%	40.5%
Left	48.8%	53.7%
Rectum	33.3%	42.9%
Differentiation		
Well-Mod	40.9%	48.1%
Poor	24.3%	29.7%
T stage		
1	100.0%	100.0%
2	66.7%	75.0%
3	45.6%	50.0%
4	24.0%	34.4%
Serum CRP		
Normal	51.0%	59.4%
High	28.9%	33.0%
Serum albumin		
Normal	48.0%	54.1%
Low	19.3%	26.3%
Klintrup score		
Good	33.0%	40.2%
Poor	58.8%	64.7%
MSI status		
Stable	40.2%	46.9%
Unstable	19.0%	28.6%
Cytoplasmic MMP-9		
Low	41.3%	51.6%
High	35.3%	38.7%
Cytoplasmic FGR		
Low	40.0%	47.2%
High	32.0%	38.0%
Nuclear FGR		
Low	36.0%	44.6%
High	45.8%	47.5%
Cytoplasmic HCK		
Low	39.2%	44.9%
High	36.2%	46.4%
Cytoplasmic FAK (tyr 861)		
Low	38.5%	45.6%
High	38.2%	44.7%

Appendix 3.5: Studied variables' 5 year recurrence-free and overall survival rates in patients with CI colorectal cancer (n=592) – Validation cohort (cohort 2)

Biomarkers	5 Year recurrence-free survival	5 Year overall survival
Sex		
Female	53.8%	58.5%
Male	53.3%	57.1%
Presentation		
Elective	60.0%	63.4%
Emergency	38.5%	44.7%
Tumour Site		
Colon	53.1%	56.8%
Rectum	55.1%	61.0%
Tumour site enhanced		
Right	48.9%	53.2%
Left	57.8%	61.0%
Rectum	54.4%	60.3%
Differentiation		
Well-Mod	55.1%	59.6%
Poor	35.4%	37.5%
T stage		
1	73.9%	73.9%
2	71.2%	75.0%
3	57.3%	61.1%
4	35.1%	41.1%
N stage		
0	61.6%	64.3%
1	44.0%	52.7%
2	29.9%	32.8%
TNM stage (simplified)		
I	72.5%	74.7%
II	58.1%	61.0%
III	40.2%	46.9%
Serum CRP		
Normal	64.9%	71.0%
High	44.3%	44.3%
Serum albumin		
Normal	60.7%	65.3%
Low	36.2%	40.2%
Klintrup score		
Good	68.8%	72.8%
Poor	47.3%	51.6%
Cytoplasmic MMP-9		
Low	55.4%	60.4%
High	51.7%	55.1%
Cytoplasmic FGR		
Low	52.8%	57.1%
High	56.3%	60.3%
Nuclear FGR		
Low	53.4%	58.7%
High	54.0%	54.7%

Cytoplasmic HCK		
Low	54.7%	58.9%
High	50.0%	54.2%
Cytoplasmic FAK (tyr 861)		
Low	54.2%	58.4%
High	52.2%	56.5%

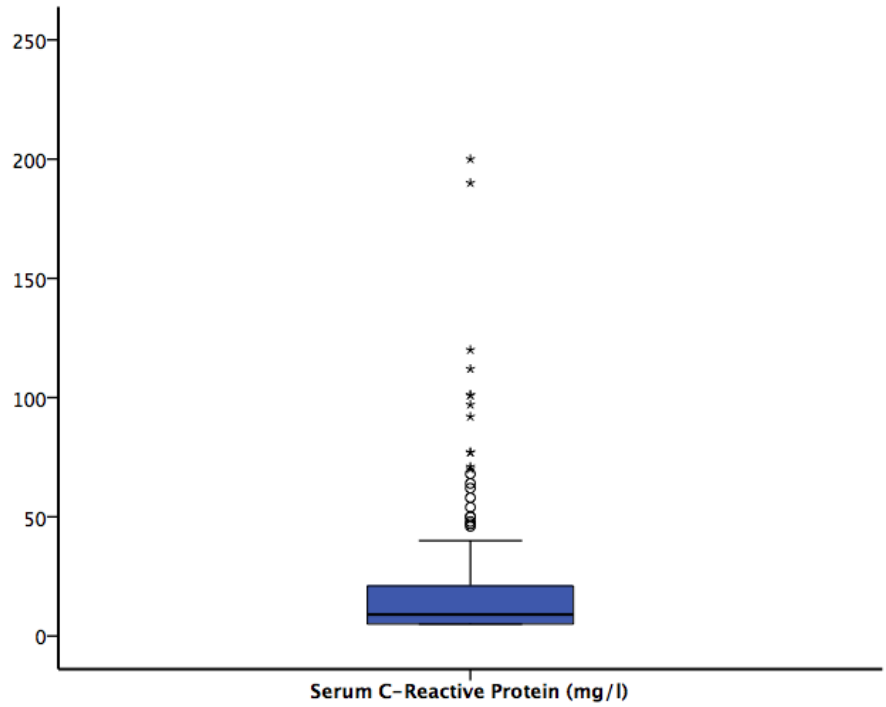
Appendix 3.7 Studied variables' 5 year recurrence-free and overall survival rates in patients colorectal cancer (n=95) – Serum and tissue cohort (cohort 3)

Biomarkers	5 Year recurrence-free survival	5 Year overall survival
Sex		
Female	56.4%	58.2%
Male	43.3%	50.0%
Presentation		
Elective	63.5%	66.7%
Emergency	18.2%	22.7%
Tumour Site		
Colon	51.3%	53.8%
Rectum	57.1%	71.4%
Tumour site		
enhanced	53.6%	55.1%
Right	40.0%	50.0%
Left	50.0%	66.7%
Rectum		
Differentiation		
Well-Mod	55.2%	58.2%
Poor	38.9%	44.4%
T stage		
1	75.0%	75.0%
2	72.7%	81.8%
3	52.5%	52.5%
4	40.0%	46.7%
N stage		
0	62.5%	64.1%
1	18.8%	31.2%
2	20.0%	20.0%
TNM stage		
(simplified)	71.4%	71.4%
I	60.0%	60.0%
II	19.0%	28.6%
III		
Serum CRP		
Normal	63.6%	68.2%
High	47.7%	50.0%
Serum albumin		
Normal	70.5%	72.7%
Low	21.7%	26.1%
Klintrup score		
Good	54.5%	63.6%
Poor	50.0%	50.0%

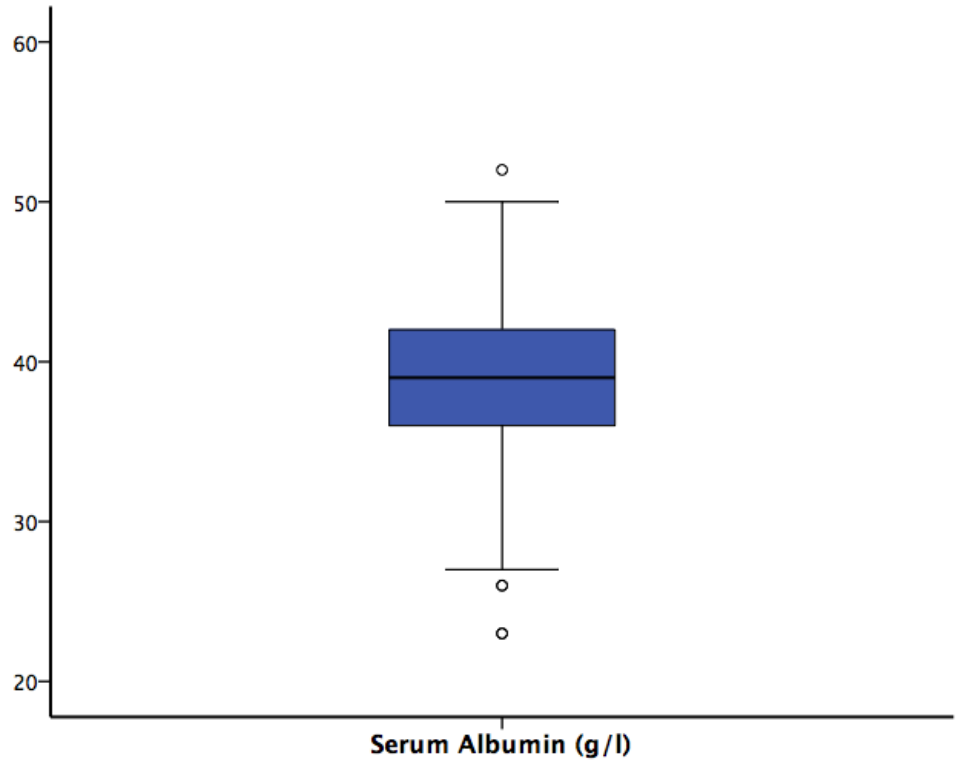
Appendix 4: Reproducibility of histologically quantified biomarkers

Protein	**Nuclear**	**Cytoplasm**	**Membrane**
MMP-9	N/A	0.93	N/A
Src416	0.92	0.86	N/A
FAK861	0.89	0.84	N/A
HCK	0.82	0.84	0.76
FGR	0.76	0.81	0.76
FYN	0.79	0.89	0.76
LCK	0.82	0.83	0.79
MLH1	0.79	N/A	N/A
MSH2	0.80	N/A	N/A
MSH6	0.81	N/A	N/A
PMS2	0.88	N/A	N/A

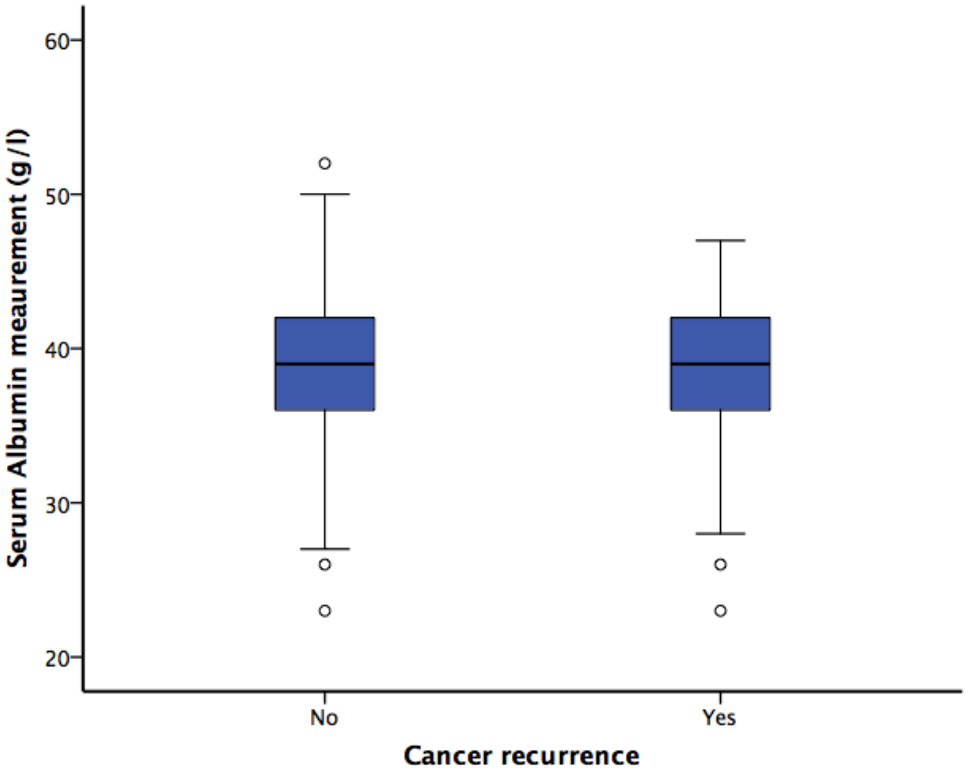
Appendix 5. 1 - Supplementary graphs for chapter 3



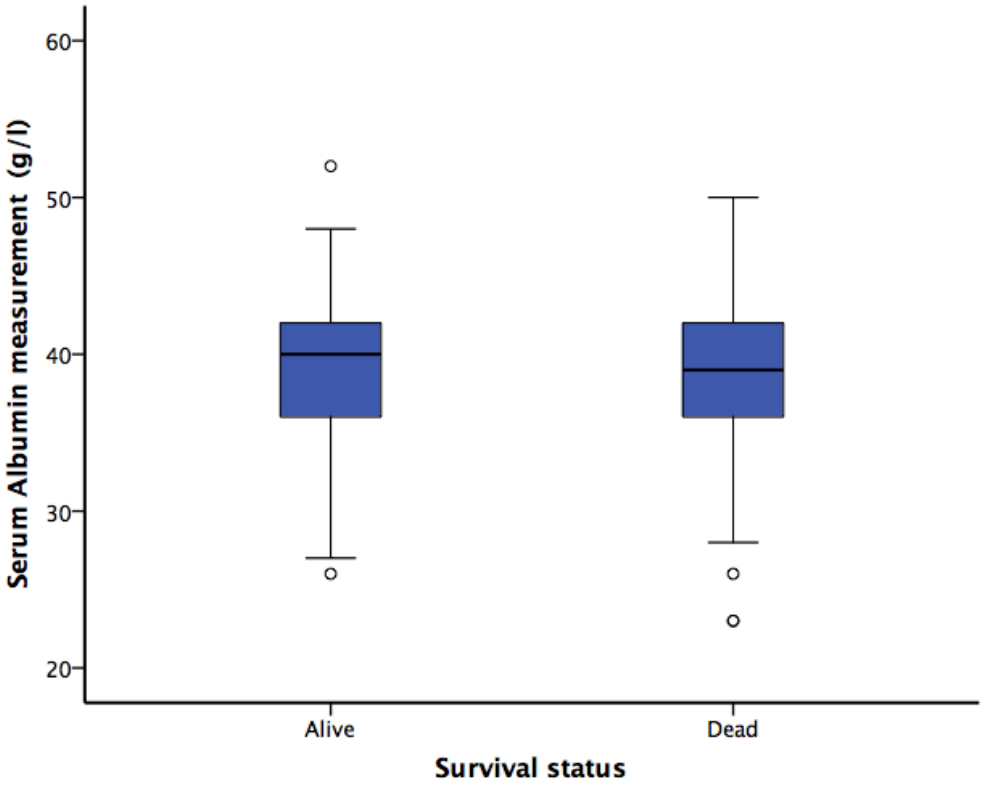
Supplementary figure 3.1: Distribution of measurements for serum CRP.



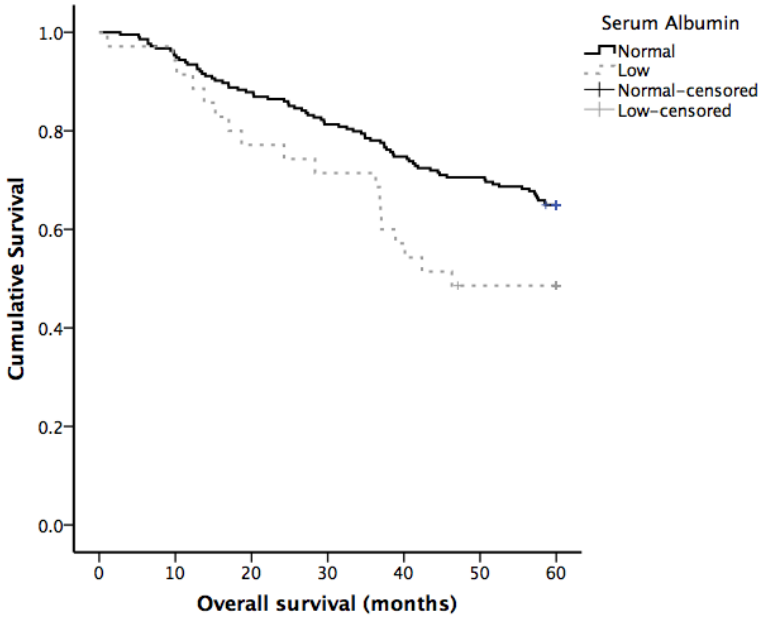
Supplementary figure 3.2: Distribution of measurements of serum albumin in patients with colorectal cancer.



Supplementary figure 3.3: The distribution of serum albumin measurements in patients with and without disease recurrence (p=0.367)

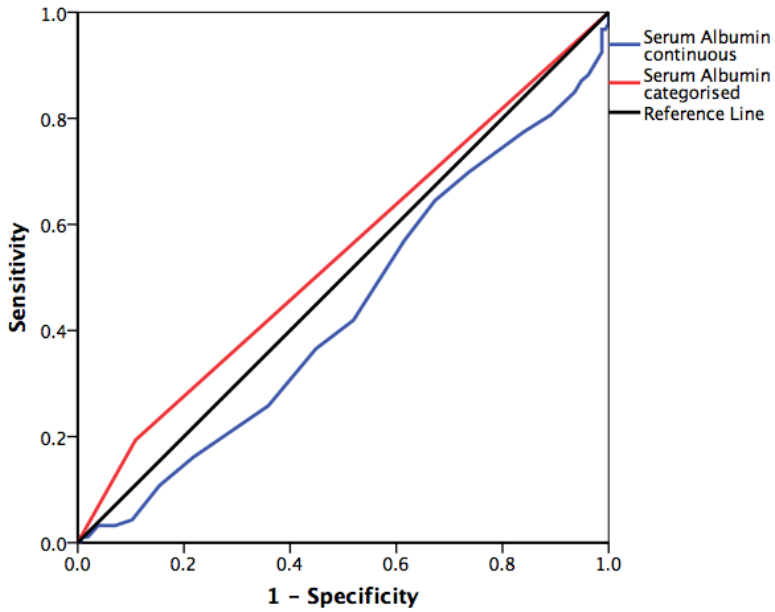


Supplementary figure 3.4: The distribution of serum albumin measurements in patients stratified by survival status (p=0.167)

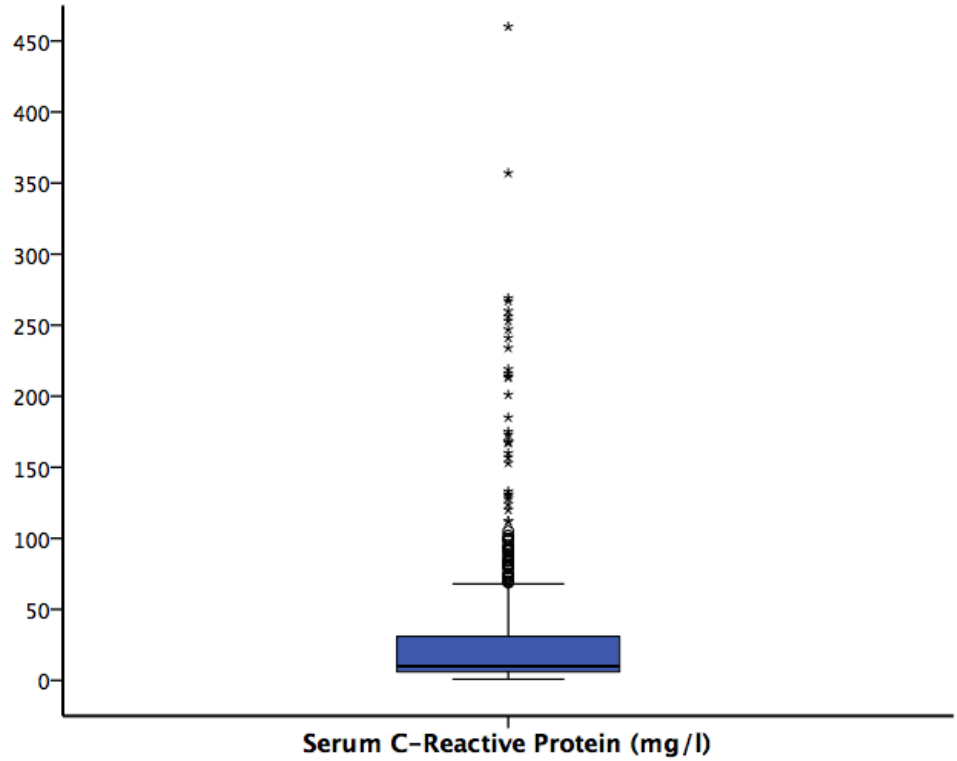


No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	157	148	138	128	118	110	103
High	25	23	18	17	14	12	12

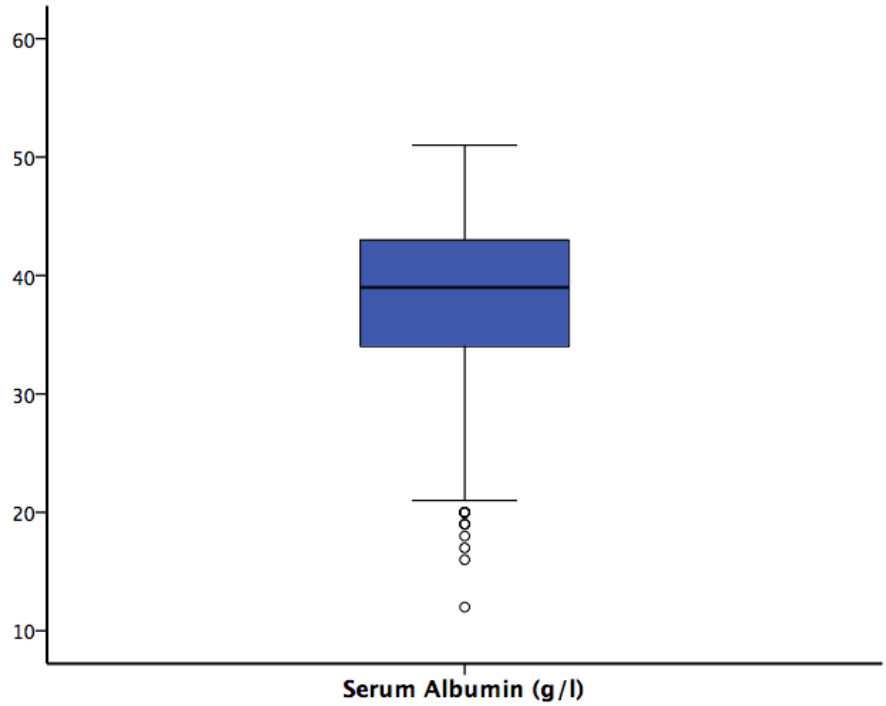
Supplementary figure 3.5: The relationship between serum albumin expression and overall survival (p=0.110)



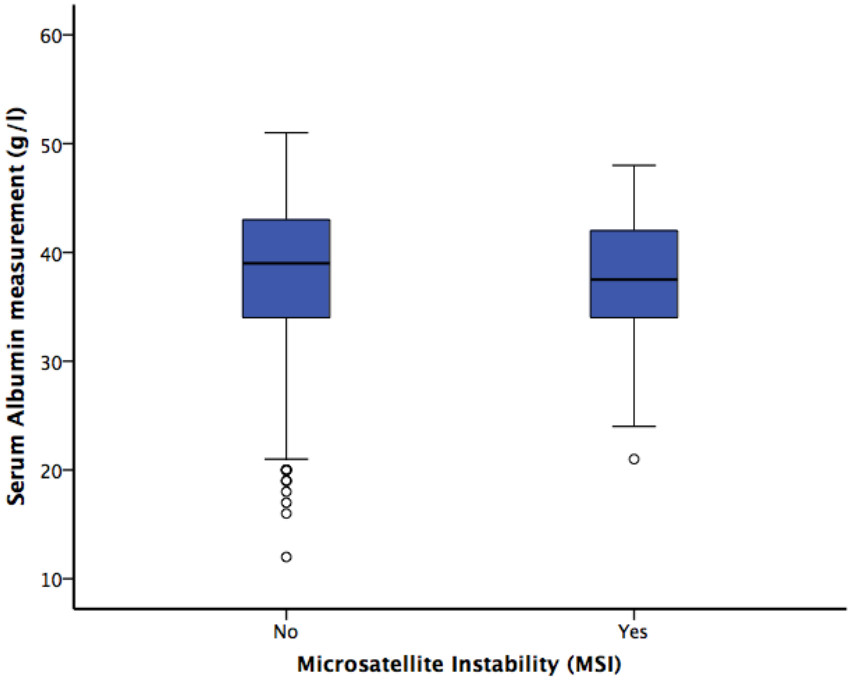
Supplementary figure 3.6 Predictive value of serum albumin in identifying patients who will die during the follow-up



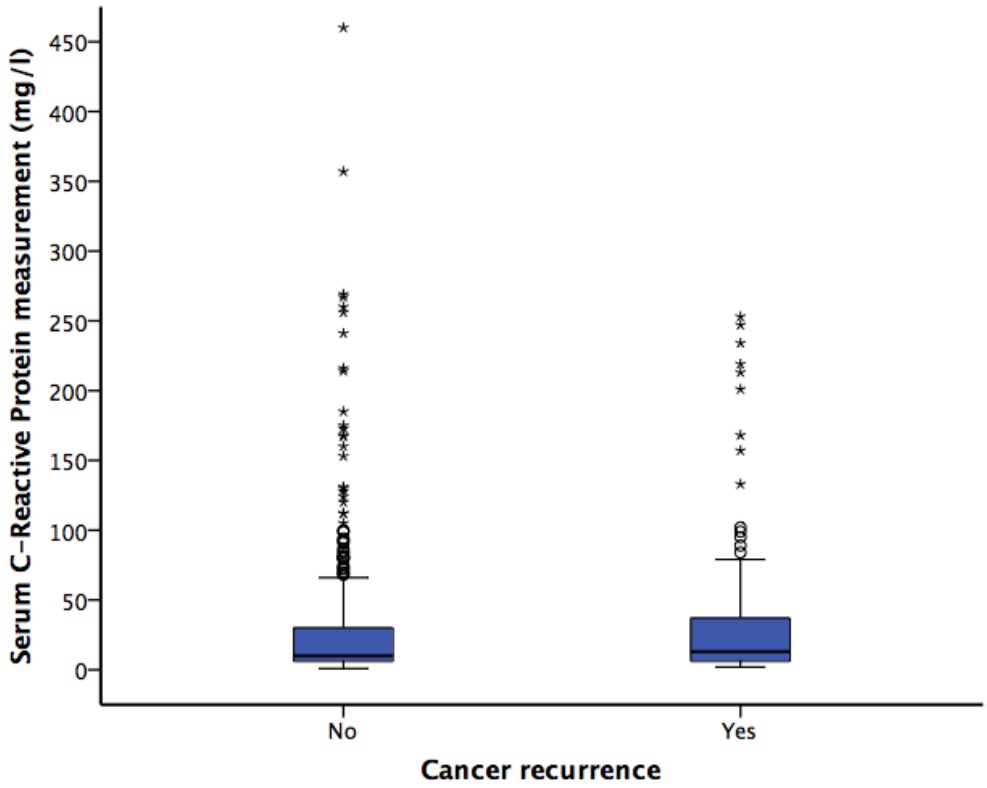
Supplementary figure 3.7: Distribution of measurements for serum CRP in patients with colorectal



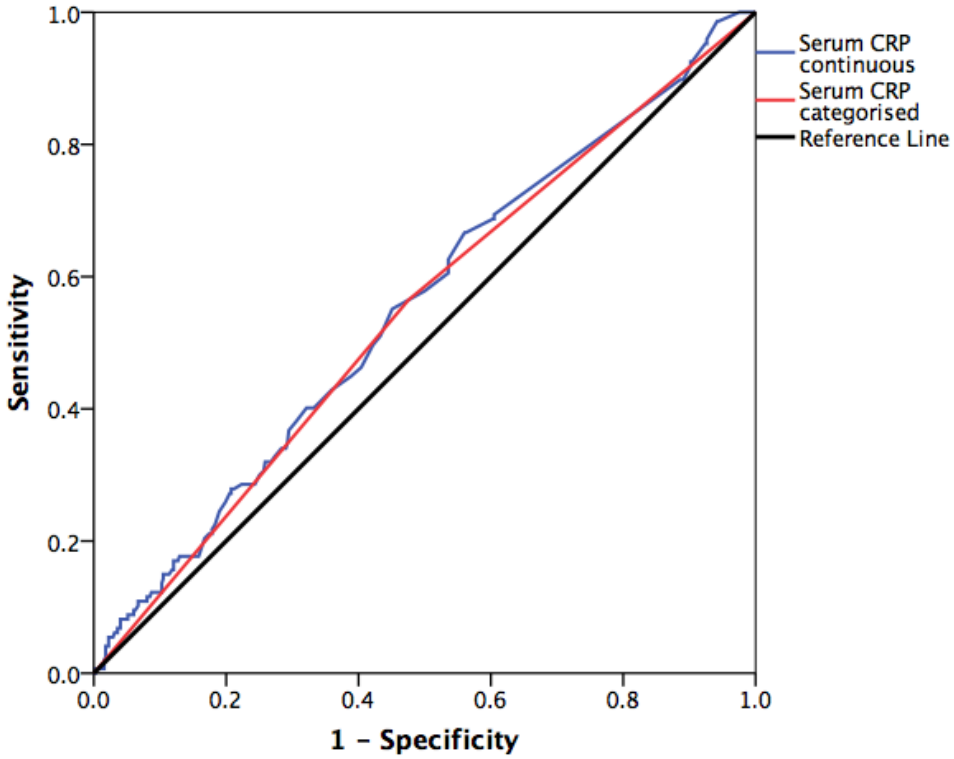
Supplementary figure 3.8: Distribution of measurements of serum albumin in patients with colorectal cancer



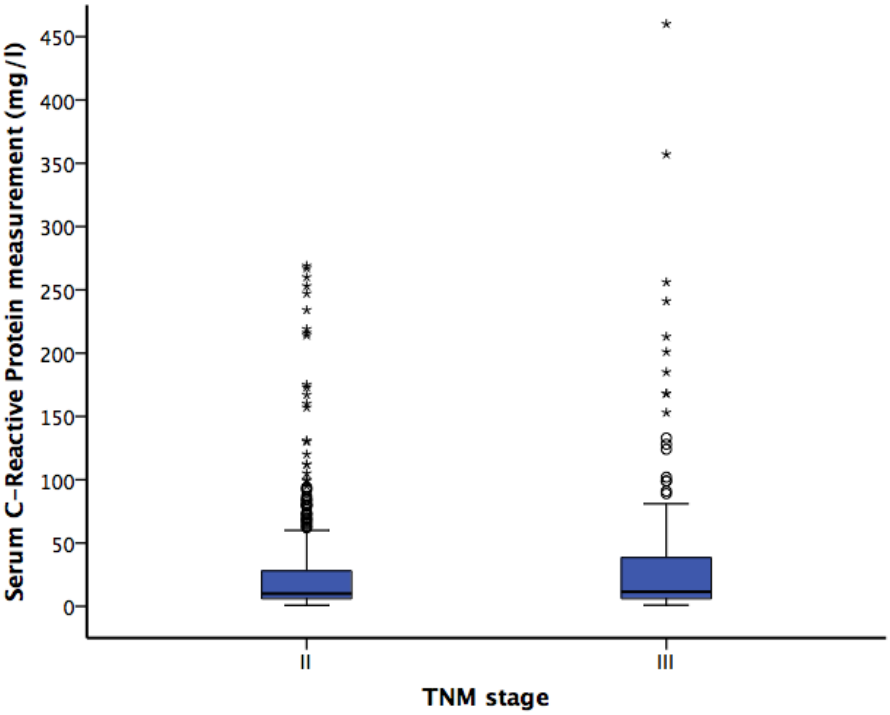
Supplementary figure 3.9: The distribution of serum albumin measurements in patients stratified by MSI status (p=0.156)



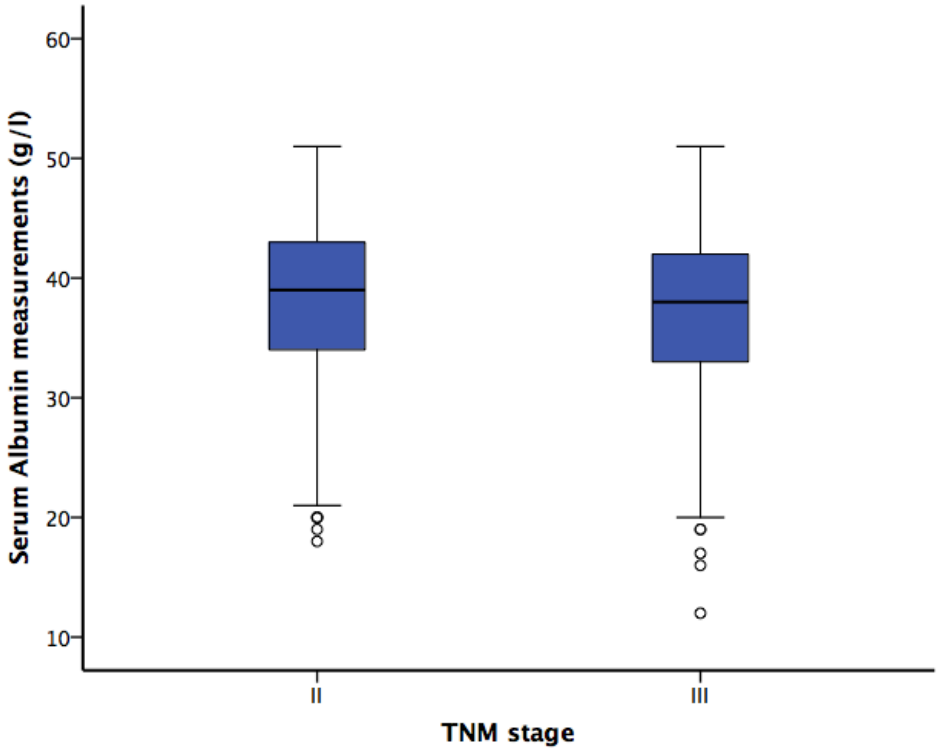
Supplementary figure 3.10: The distribution of serum CRP measurements in patients with and without disease recurrence (p=0.112)



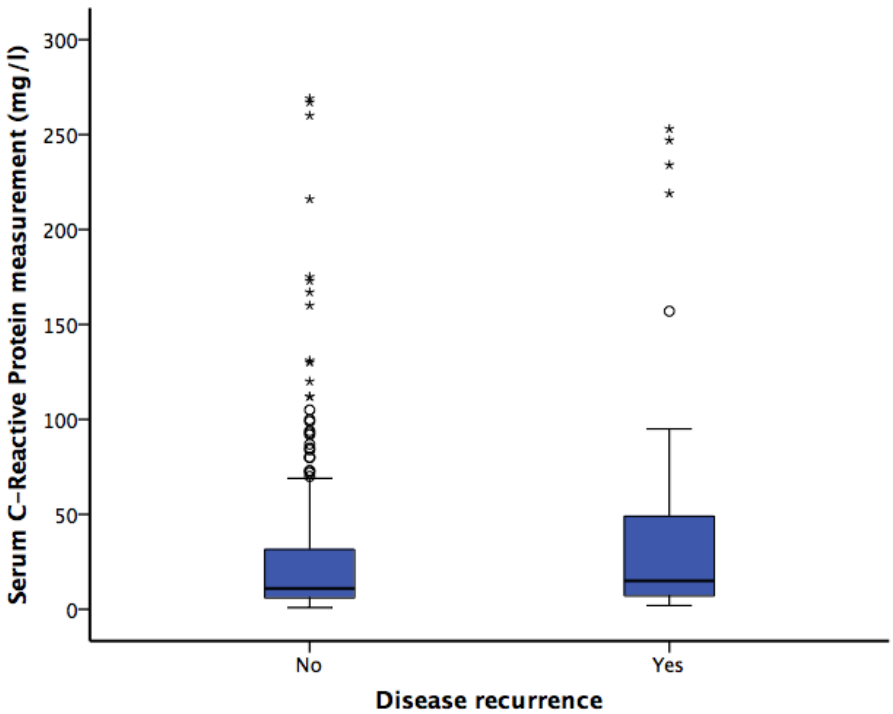
Supplementary figure 3.11: The predictive value of CRP in identifying patients who will develop cancer recurrence



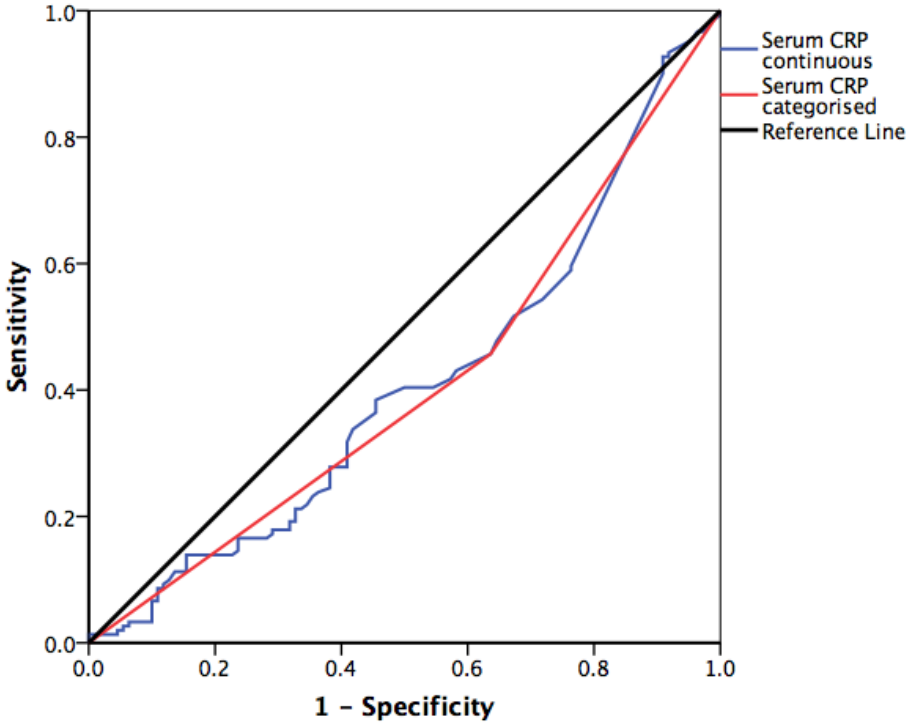
Supplementary figure 3.12: Distribution of measurements of serum CRP in patients with TNM stage II and III colorectal cancer.



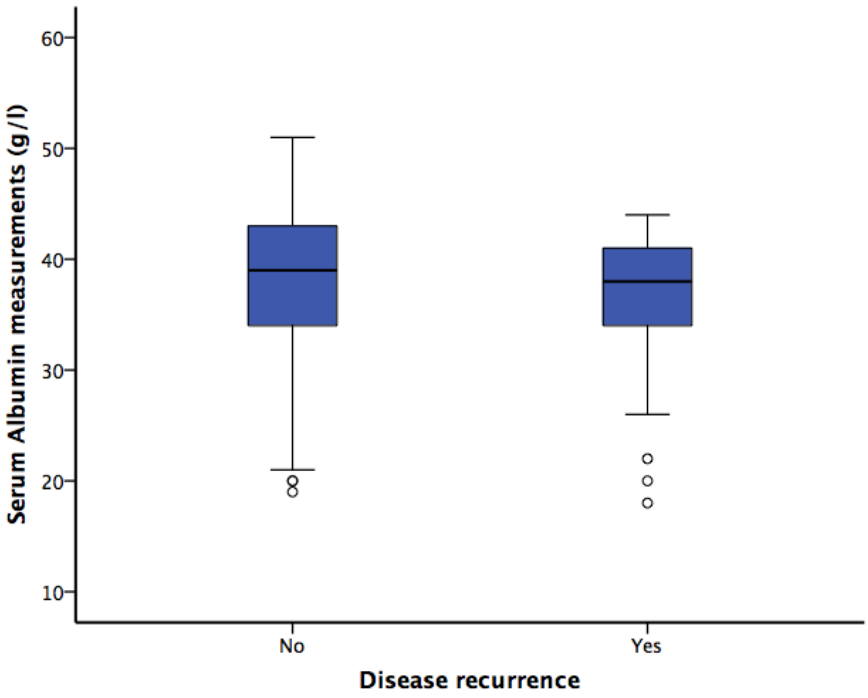
Supplementary figure 3.13: Distribution of measurements of serum albumin in patients with TNM stage II and III colorectal cancer



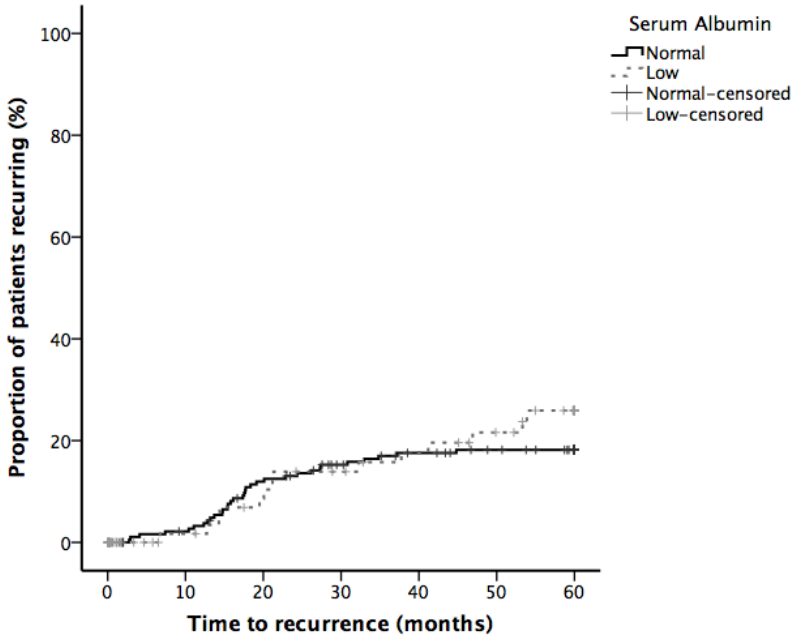
Supplementary figure 3.14: The distribution of serum CRP measurements in patients with and without disease recurrence in patients with stage II colorectal cancer (p=0.230)



Supplementary figure 3.15: The predictive value of CRP in identifying patients with stage II colorectal cancer who will develop recurrence

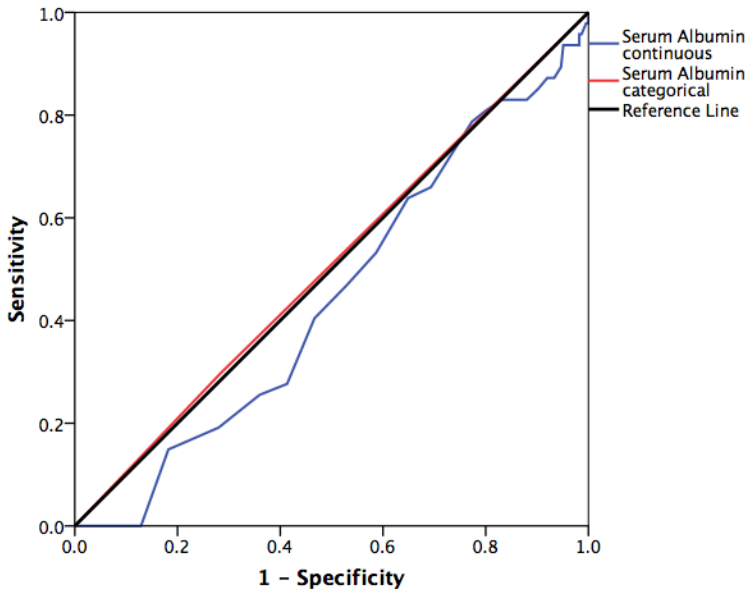


Supplementary figure 3.16: The distribution of serum albumin measurements in stage II colorectal cancer patients stratified by recurrence status (p=0.236)

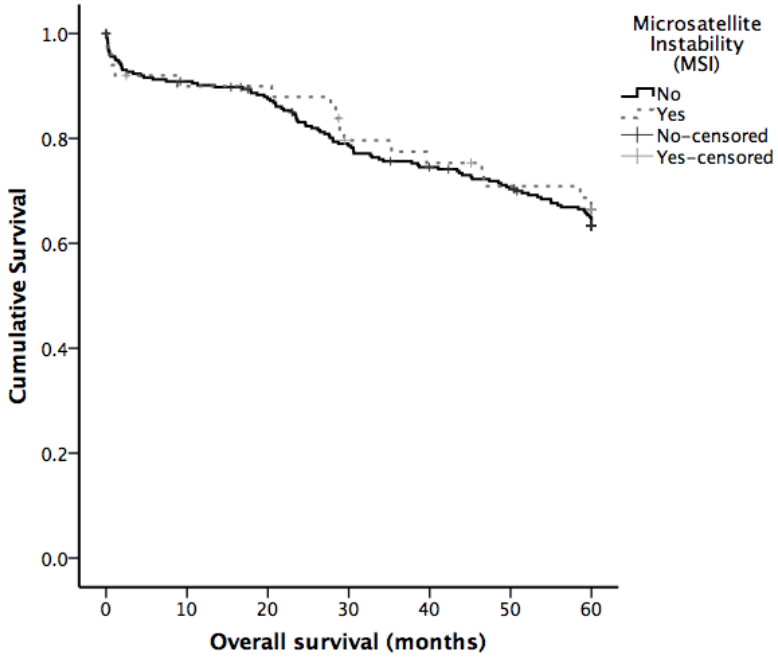


No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	194	181	161	148	140	134	128
Low	78	58	52	47	43	38	31

Supplementary figure 3.17: Serum albumin expression and recurrence-free survival in patients with stage II colorectal cancer (p=0.349)

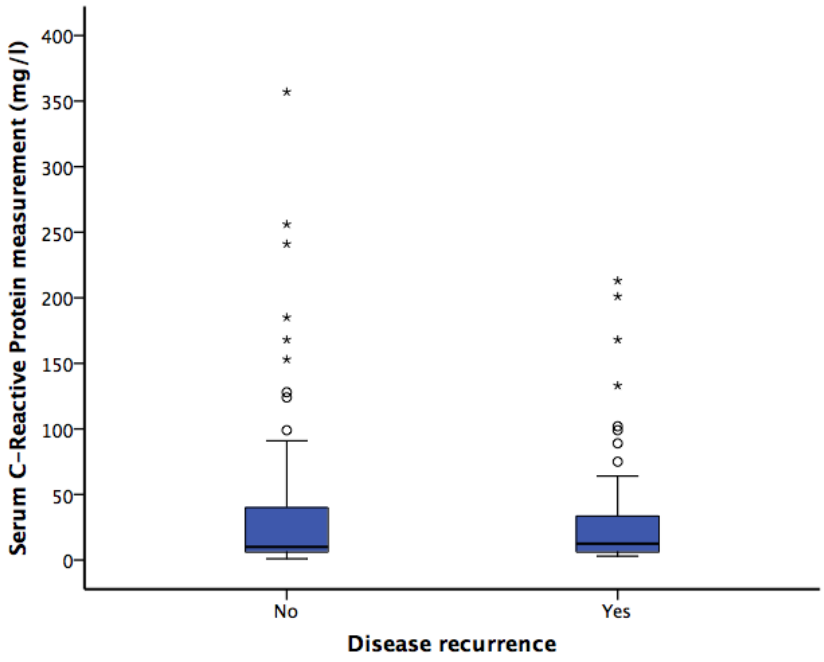


Supplementary figure 3.18: Predictive value of serum albumin in identifying patients with stage II colorectal cancer who will develop cancer recurrence

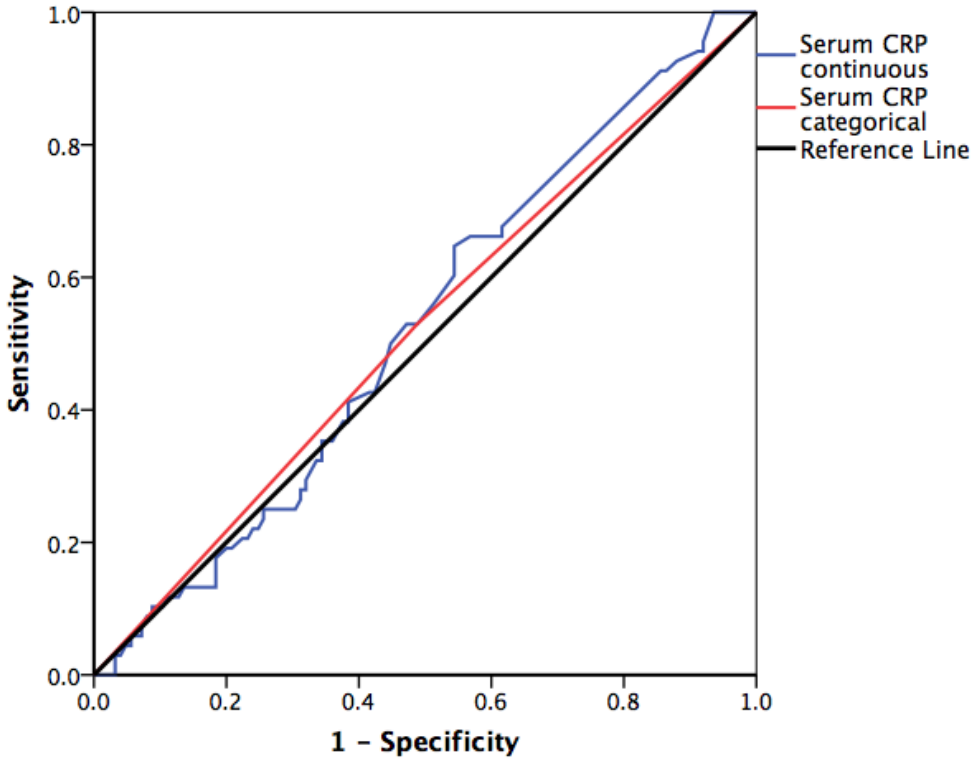


No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
MSS	277	248	236	211	198	186	169
MSI	50	44	44	37	35	32	30

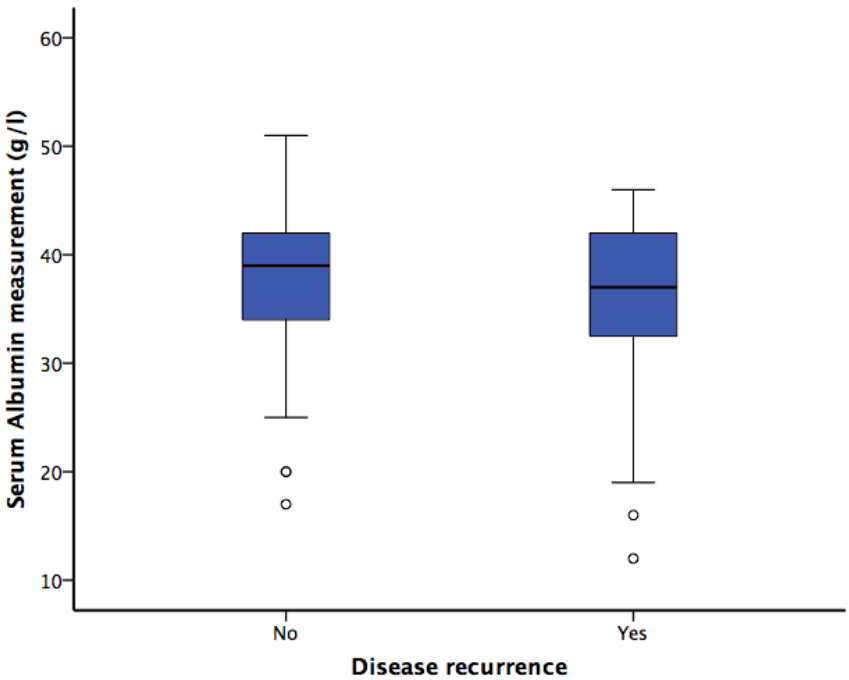
Supplementary figure 3.19: The relationship between MSI status and overall survival in stage II colorectal cancer (p=0.688)



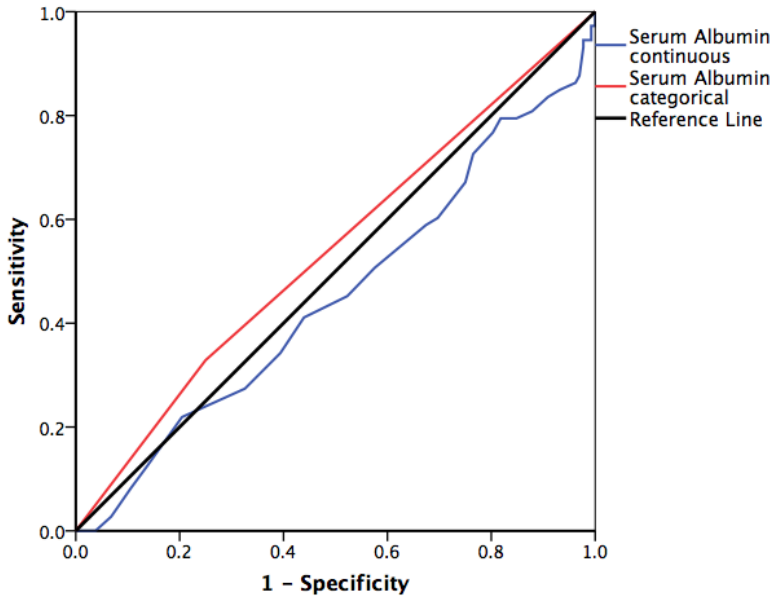
Supplementary figure 3.20: The distribution of serum CRP measurements in patients with stage III colorectal cancer stratified by cancer recurrence (p=0.557)



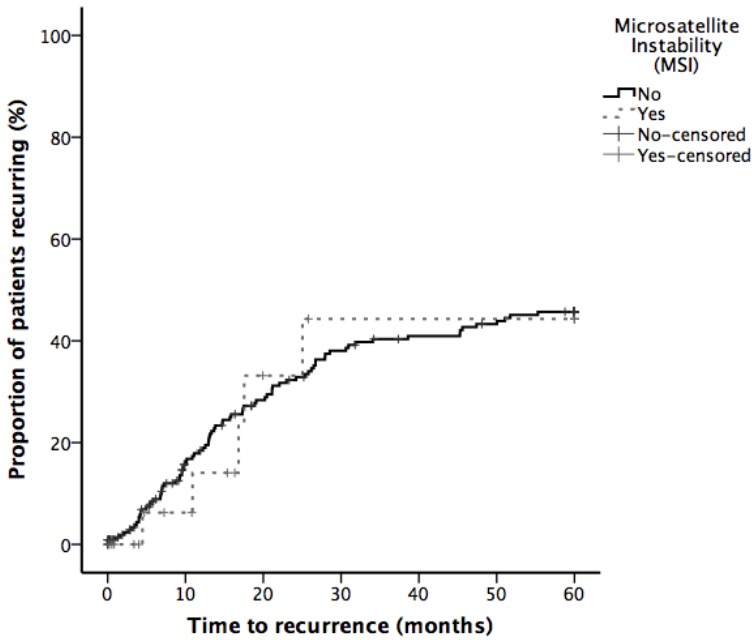
Supplementary figure 3.21: The predictive value of CRP in identifying patients with stage III colorectal cancer who developed recurrence during the 5 years follow-up



Supplementary figure 3.22: The distribution of serum albumin measurements in patients with and without cancer recurrence (p=0.256)



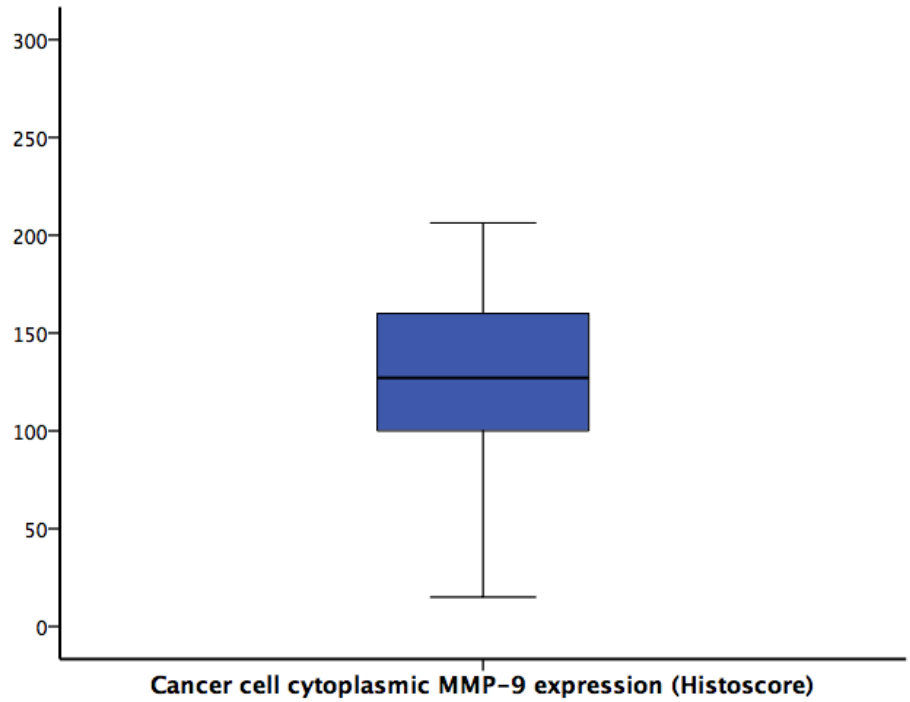
Supplementary figure 3.23: Predictive value of serum albumin in identifying patients with stage III colorectal cancer who developed cancer recurrence



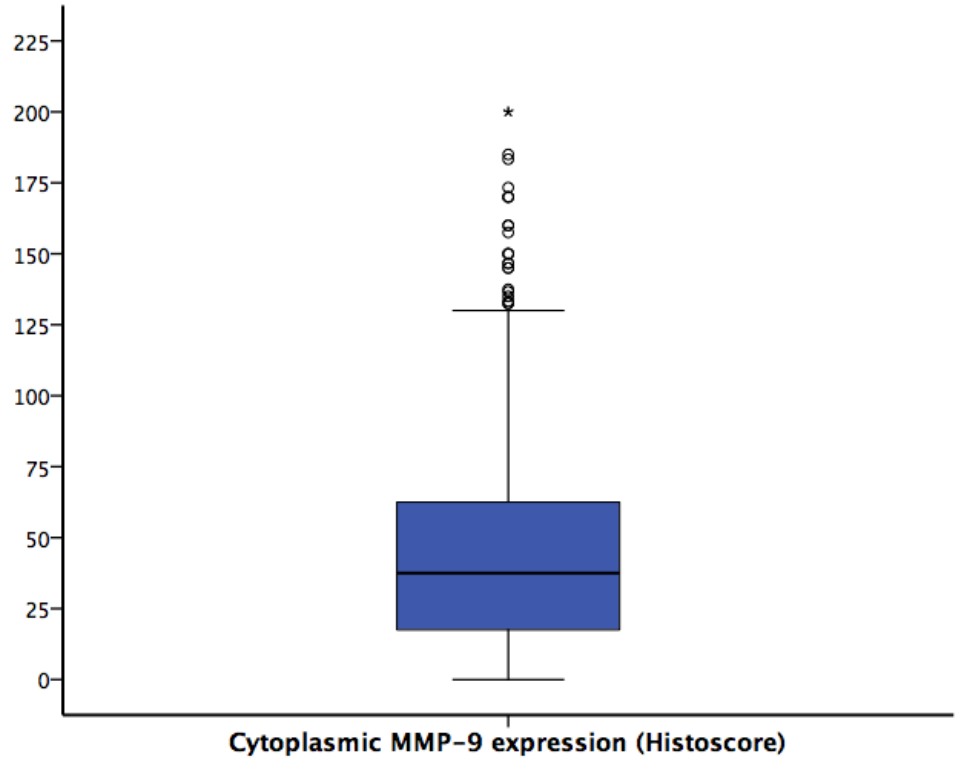
No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
MSS	224	155	127	108	100	95	90
MSI	21	13	6	4	4	4	4

Supplementary figure 3.24: The relationship between MSI status and recurrence-free survival in patients with stage III colorectal cancer (p=0.836)

Appendix 5.2 – Supplementary graphs and tables for results chapter 4

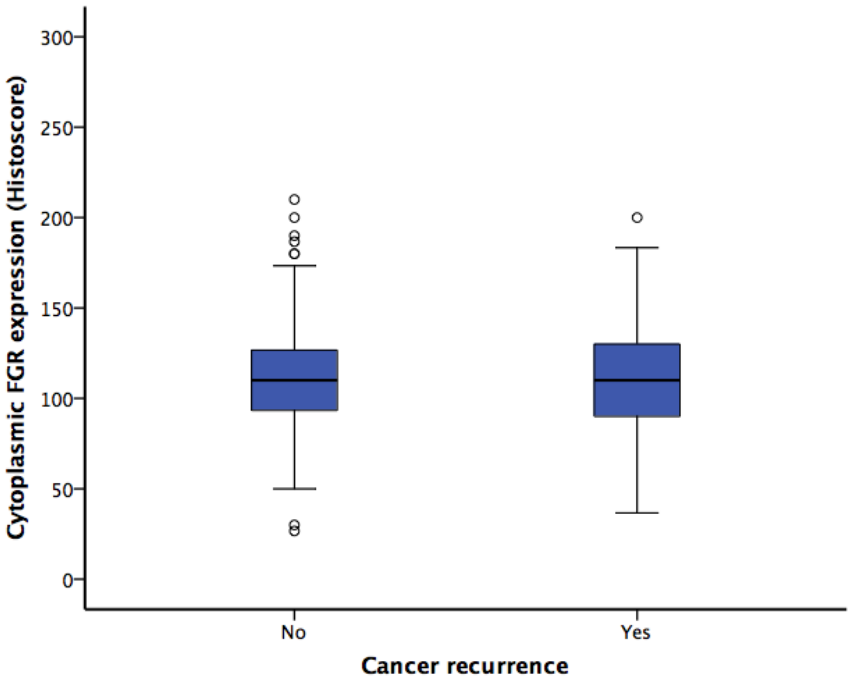


Supplementary figure 4.1: Distribution of measurements for cancer cell cytoplasmic MMP-9.

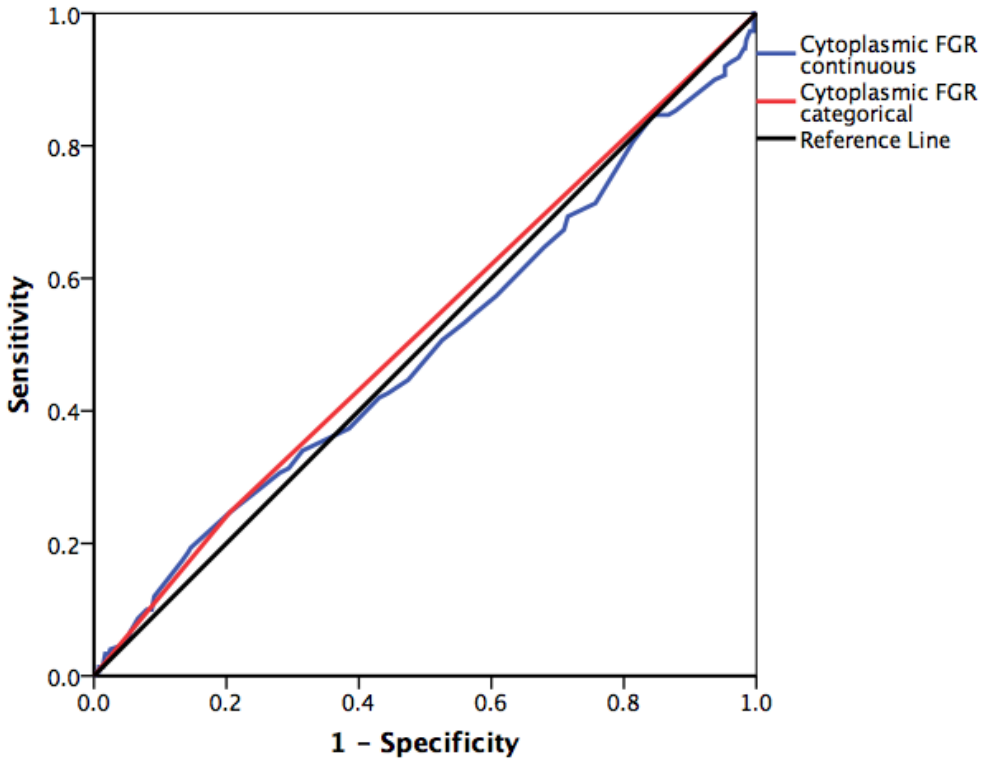


Supplementary figure 4.2: Distribution of measurements of cytoplasmic MMP-9 in patients with colorectal cancer.

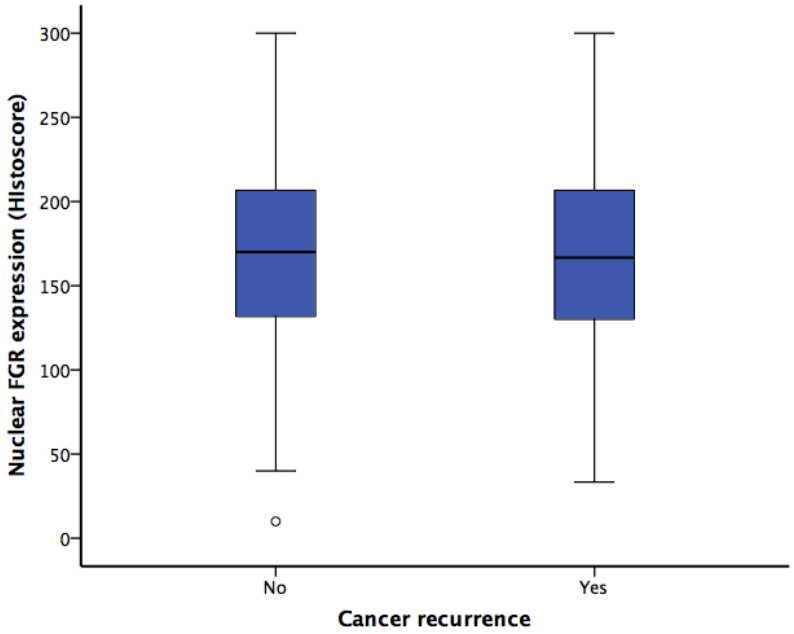
Appendix 5.3 – Supplementary graphs/tables for chapter 5



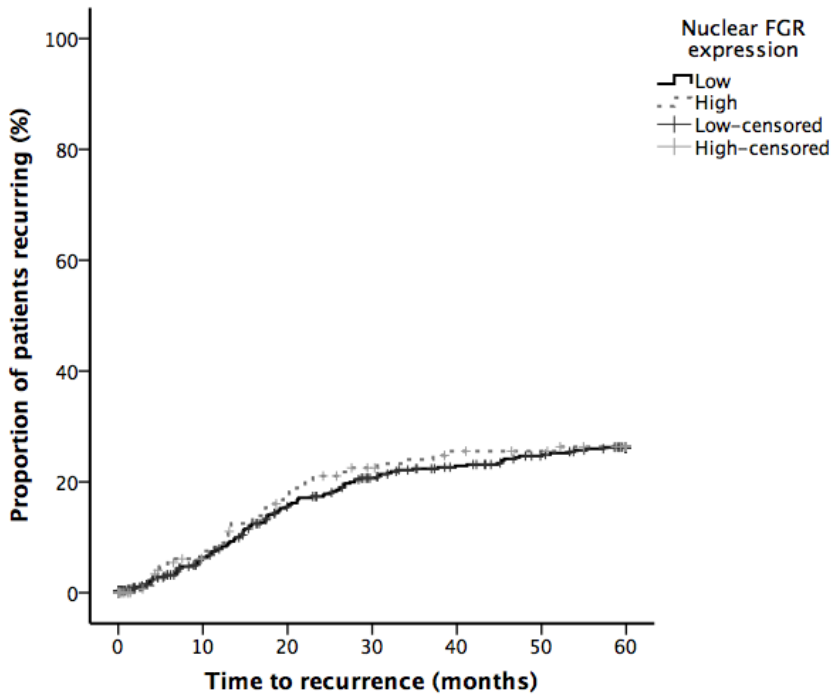
Supplementary Figure 5.1: The distribution of Cytoplasmic FGR measurements in patients with and without disease recurrence (p=0.817)



Supplementary Figure 5.2 The predictive value of cytoplasmic FGR in identifying patients who will develop cancer recurrence

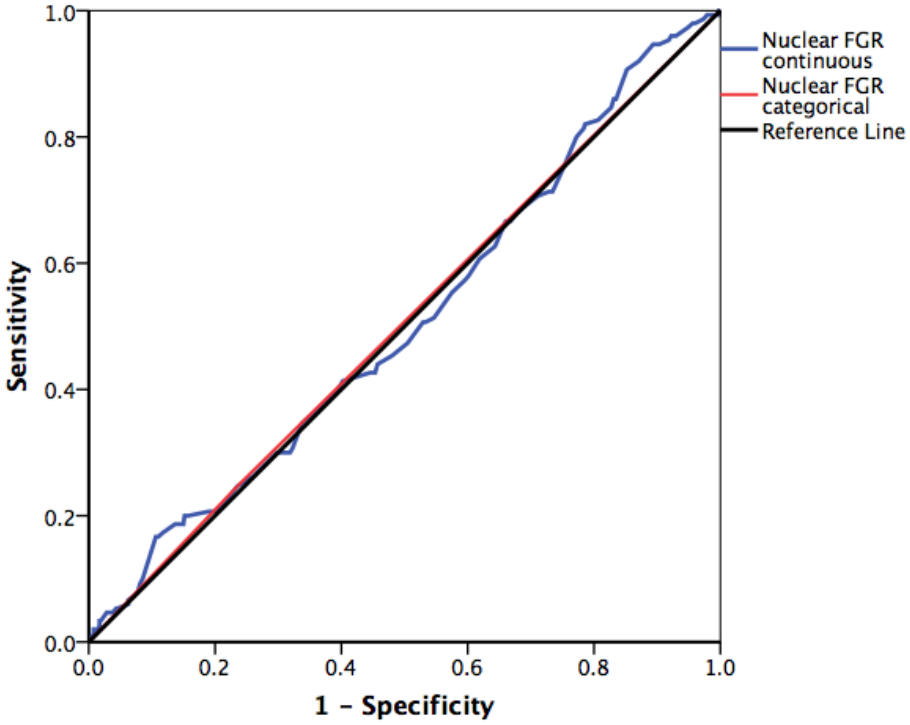


Supplementary figure 5.3: The distribution of nuclear FGR measurements in patients with and without cancer recurrence (p=0.777)

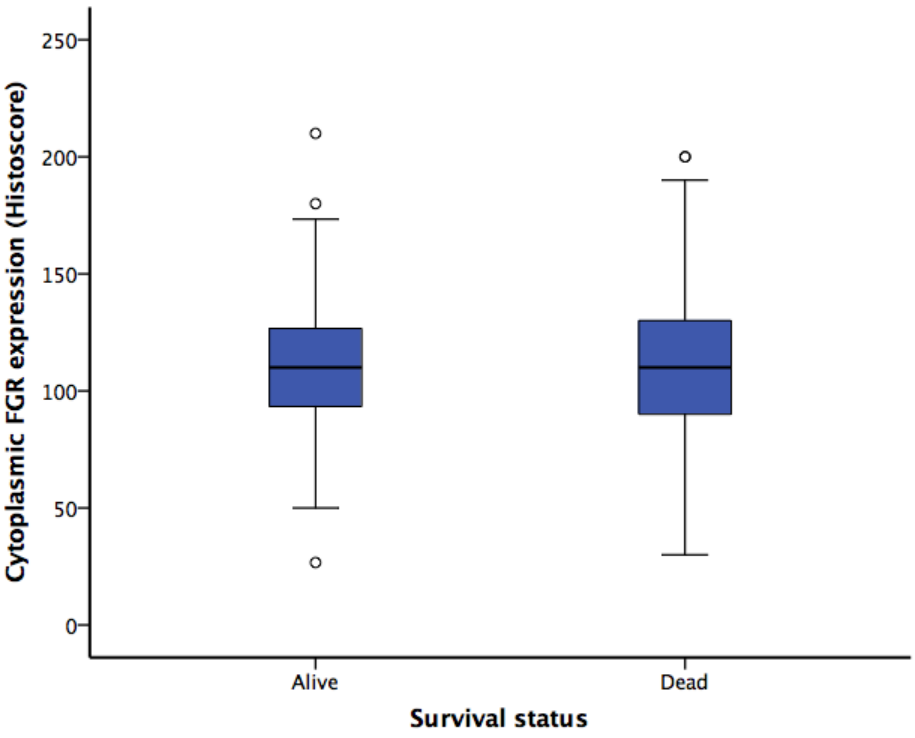


No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	516	415	361	328	307	290	272
Low	161	133	115	104	96	96	90

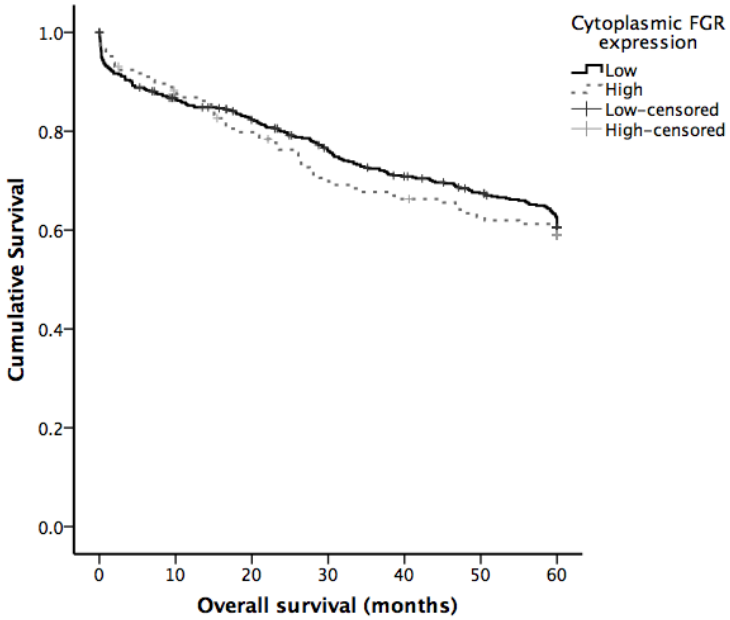
Supplementary figure 5.4: The relationship between nuclear FGR expression and recurrence-free survival



Supplementary figure 5.5: Predictive value of nuclear FGR in identifying patients who will develop cancer recurrence

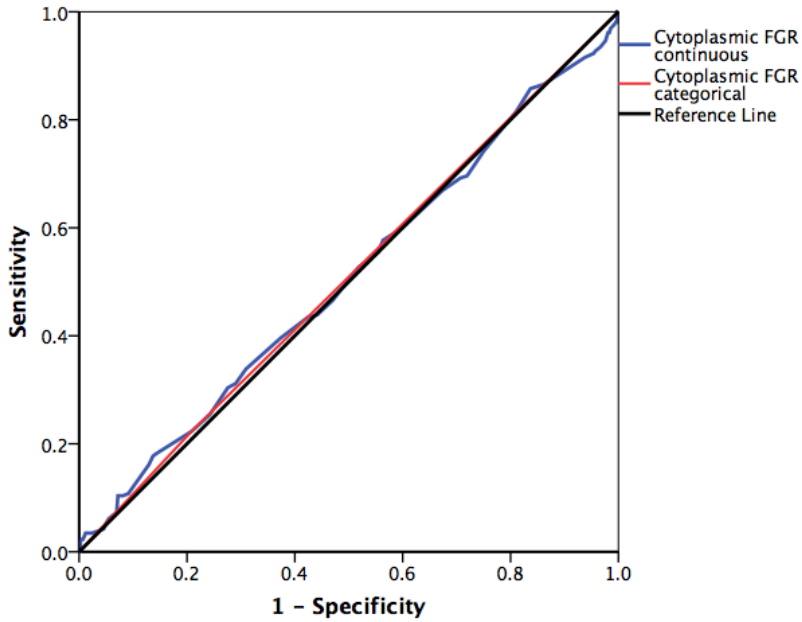


Supplementary figure 5.6: The distribution of cytoplasmic FGR measurements in patients stratified by survival status (p=0.783)

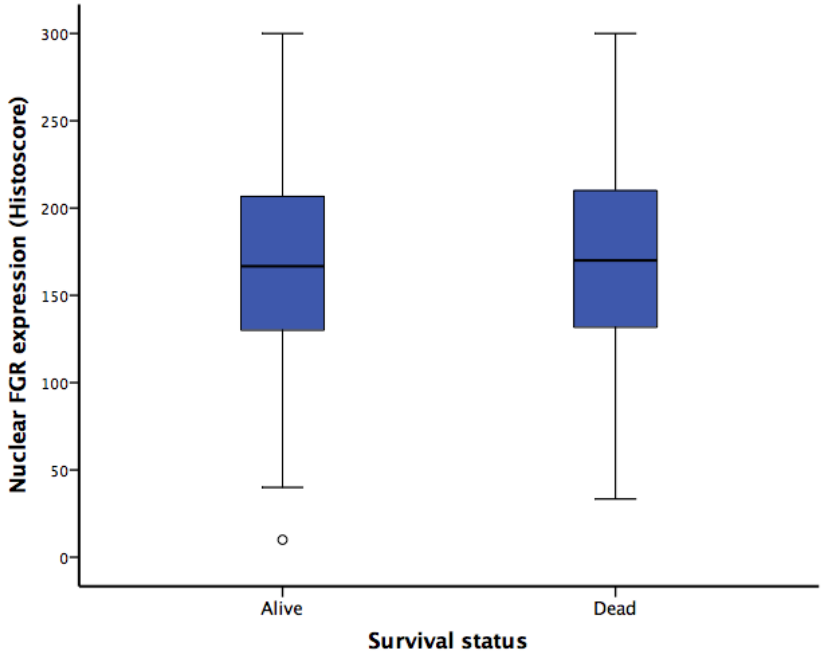


No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Low	532	451	422	383	354	332	305
High	145	126	113	99	93	87	85

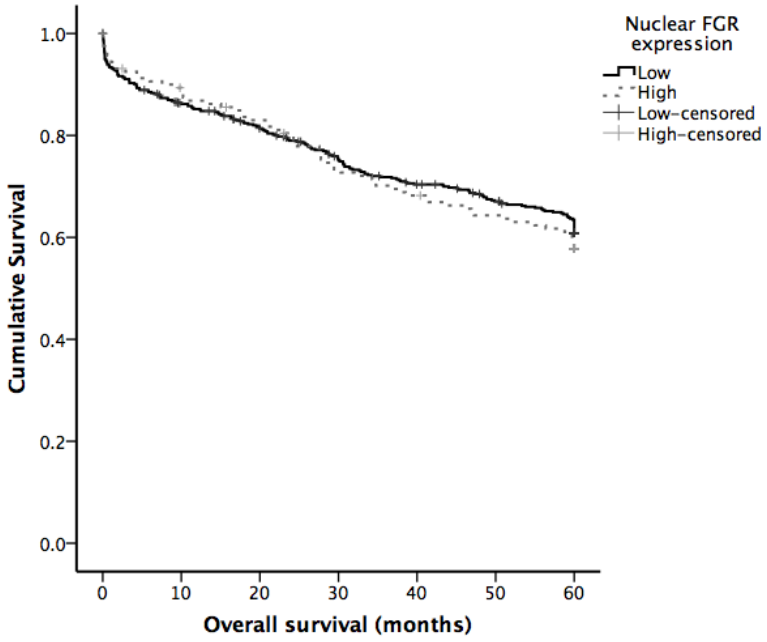
Supplementary figure 5.7: The relationship between cytoplasmic FGR expression and overall survival in patients with colorectal cancer



Supplementary figure 5.8: Predictive value of cytoplasmic FGR in identifying patients who will die during follow-up

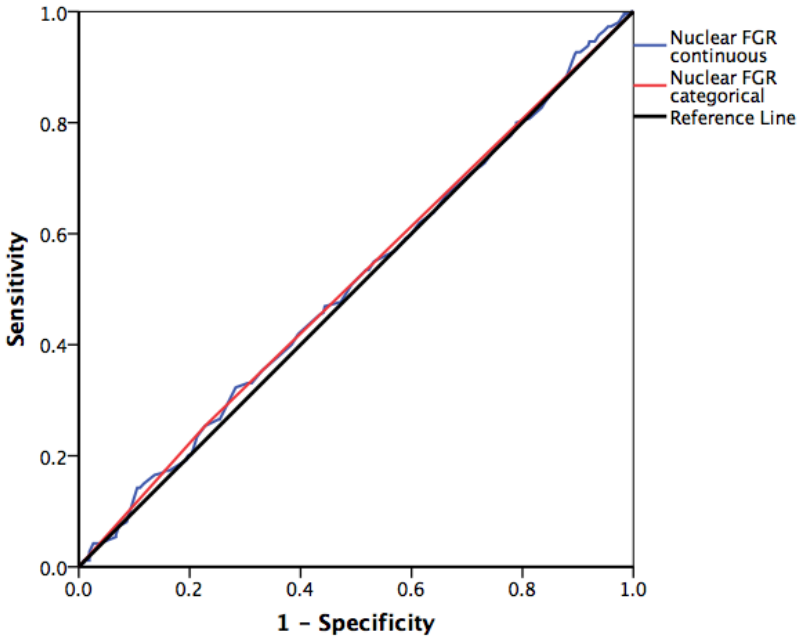


Supplementary figure 5.9: The distribution of nuclear FGR measurements in patients stratified by survival status (p=0.663)

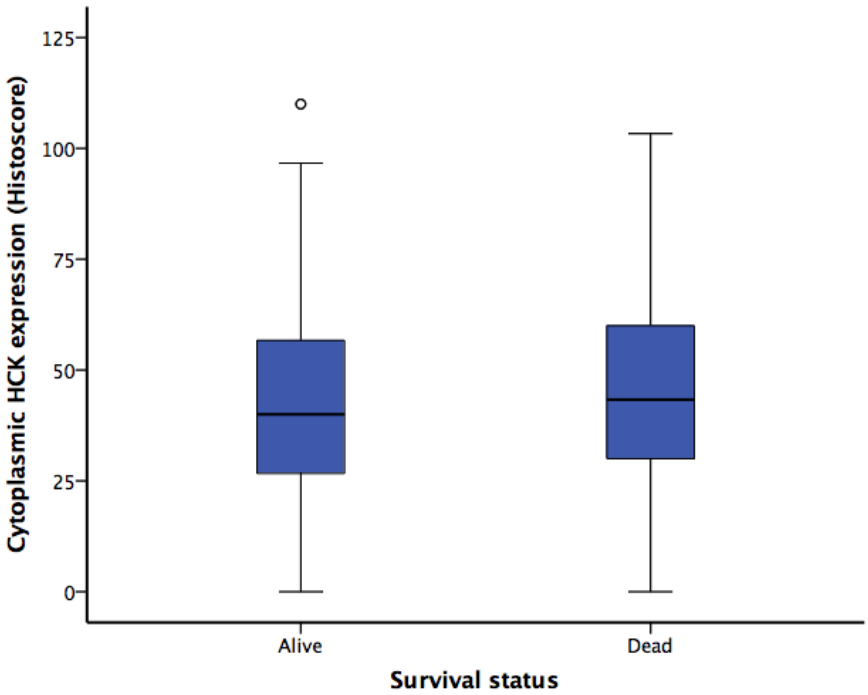


No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	516	437	405	368	341	320	300
Low	161	140	130	114	106	99	90

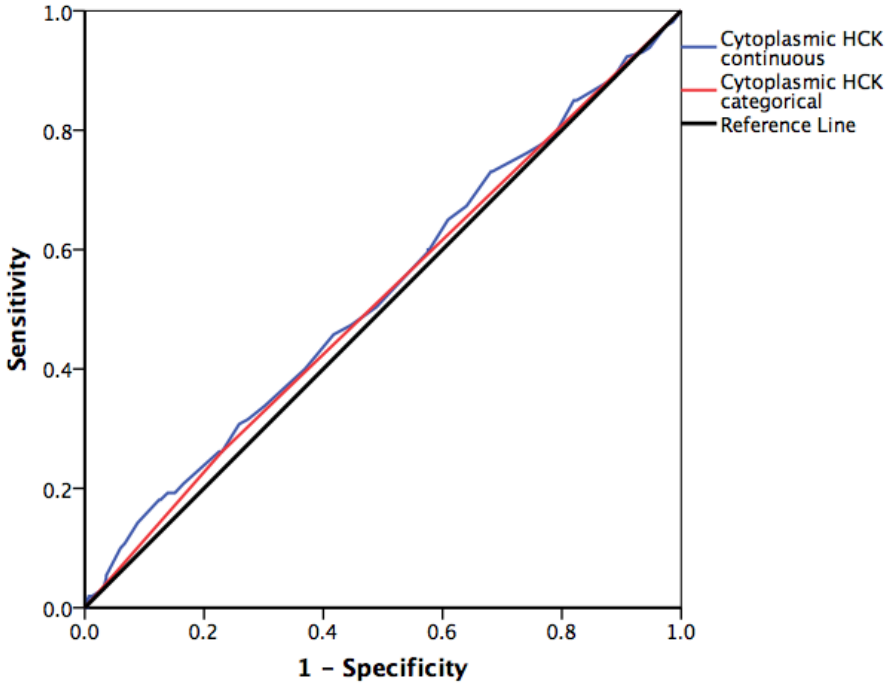
Supplementary figure 5.10: The relationship between nuclear FGR expression and overall survival in patients with colorectal cancer



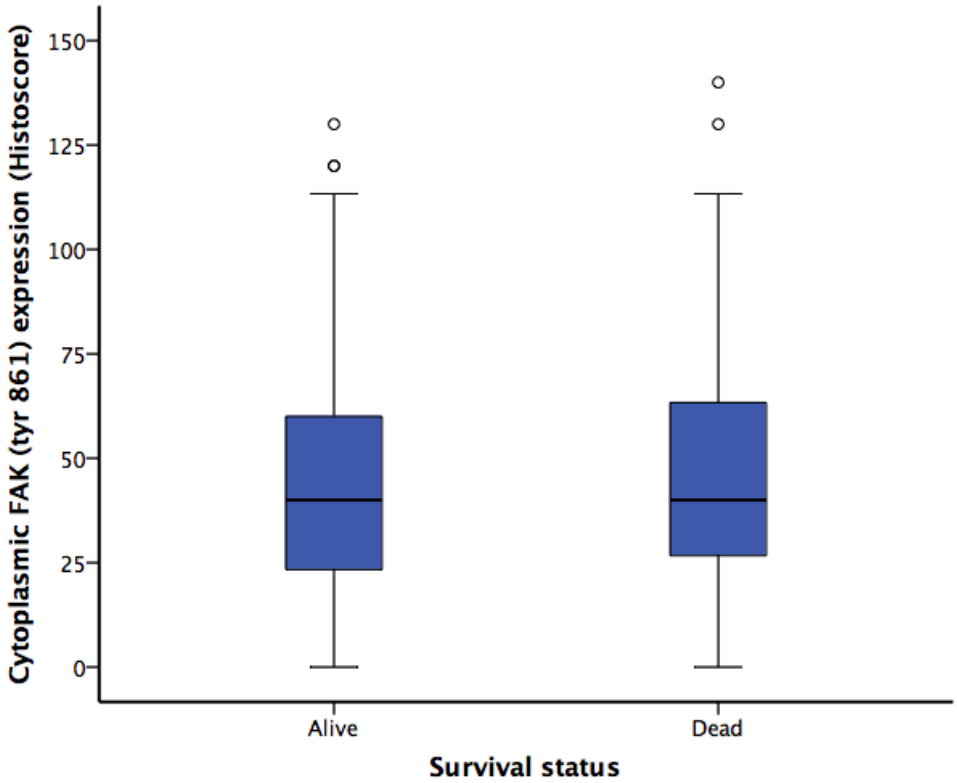
Supplementary figure 5.11: Predictive value of nuclear FGR in identifying patients who will die during the follow-up



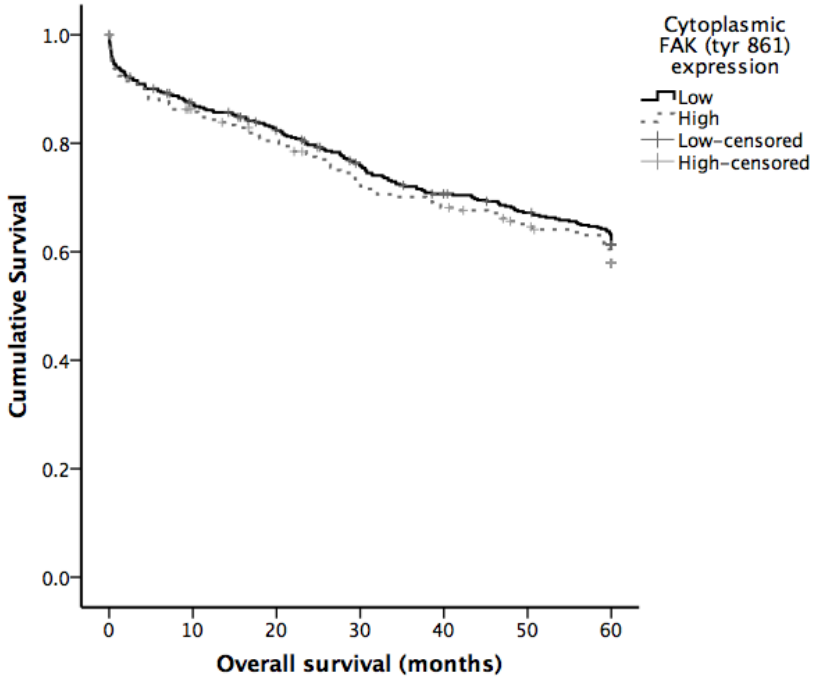
Supplementary figure 5.12: The distribution of cytoplasmic HCK measurements in patients stratified by survival status (p=0.240)



Supplementary figure 5.13: Predictive value of cytoplasmic HCK in identifying patients who will die during follow-up

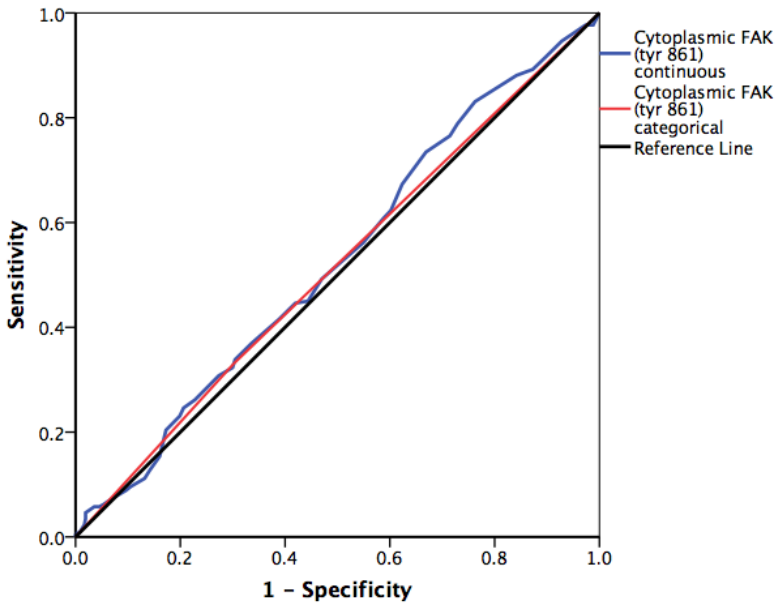


Supplementary figure 5.14: The distribution of cytoplasmic FAK (tyr 861) expression in patients stratified by survival status (p=0.246)

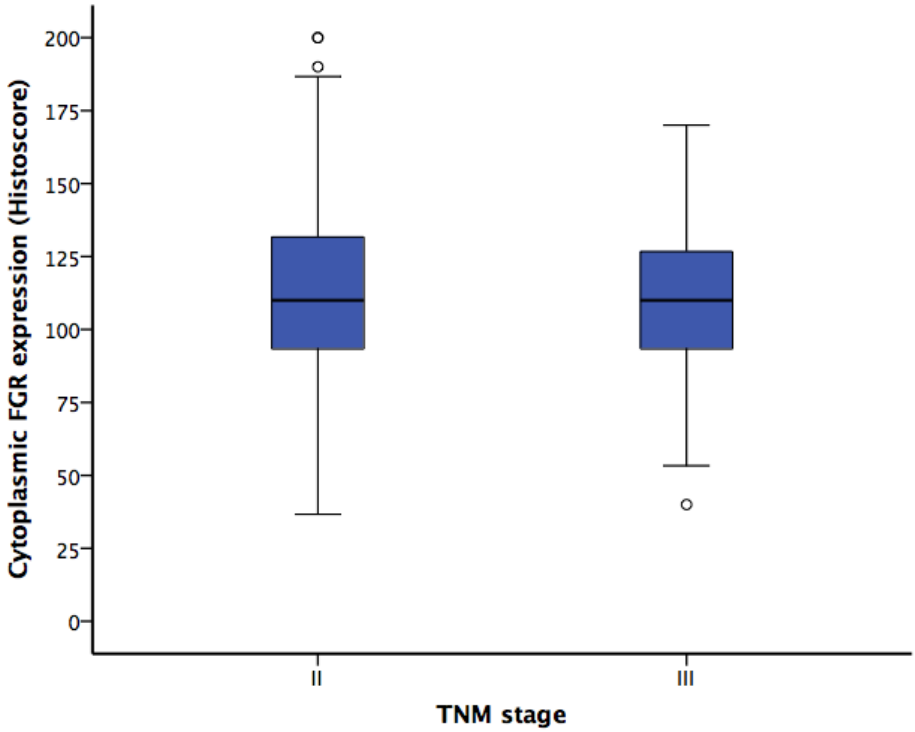


No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	465	399	370	335	309	292	273
Low	212	178	165	147	138	127	117

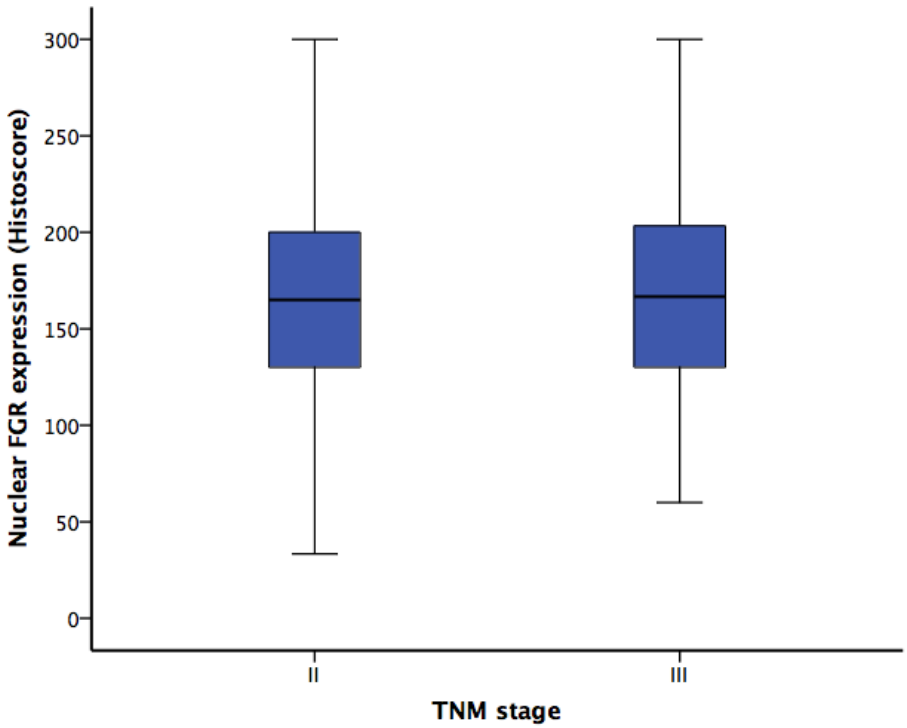
Supplementary figure 5.15: The relationship between cytoplasmic FAK (tyr 861) expression and overall survival in patients with colorectal cancer



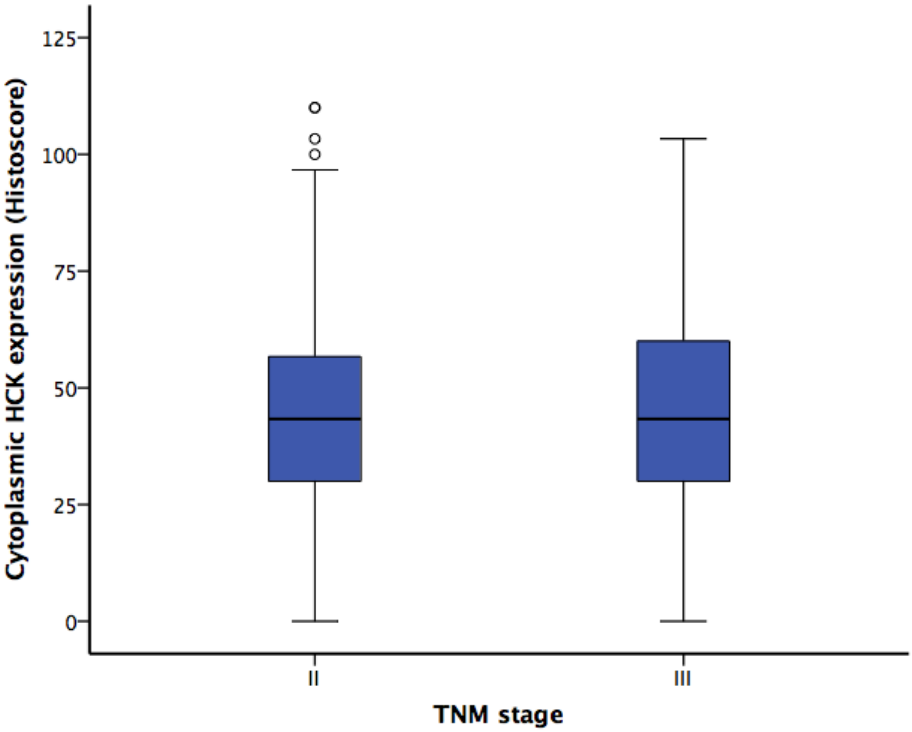
Supplementary figure 5.16: Predictive value of cytoplasmic FAK (tyr 861) in identifying patients who will die during the follow-up



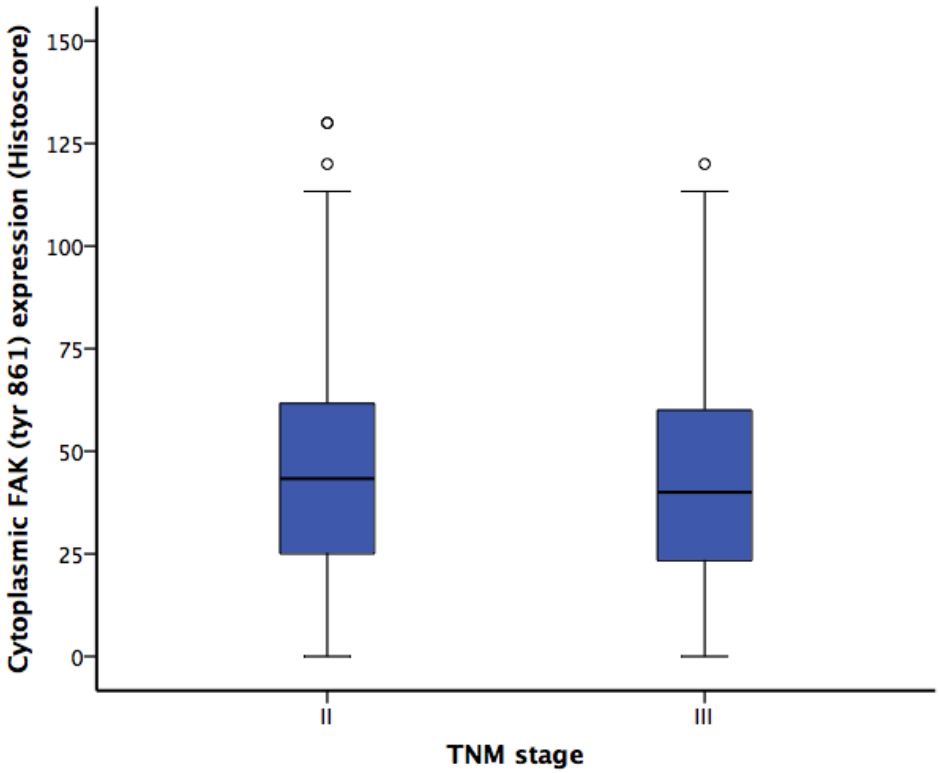
Supplementary figure 5.17: Distribution of measurements of cytoplasmic FGR expression in patients with TNM stage II and III colorectal cancer.



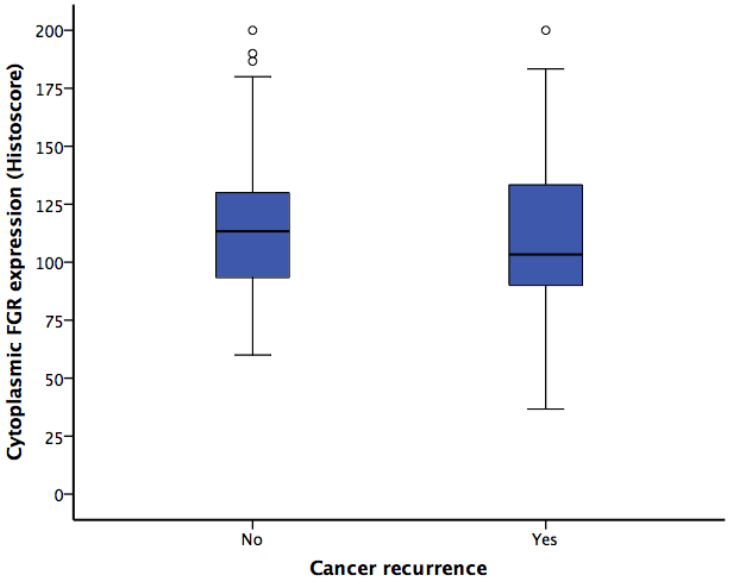
Supplementary figure 5.18: Distribution of measurements of nuclear FGR in patients with TNM stage II and III colorectal cancer.



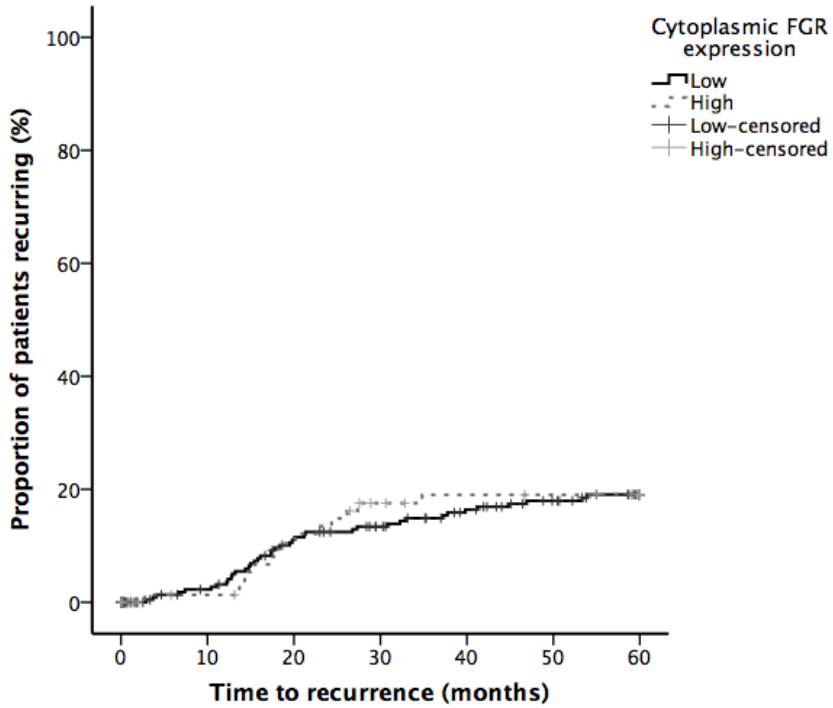
Supplementary figure 5.19: Distribution of measurements of cytoplasmic HCK in patients with TNM stage II and III colorectal cancer.



Supplementary figure 5.20: Distribution of measurements of cytoplasmic FAK (tyr 861) expression in patients with TNM stage II and III colorectal cancer.

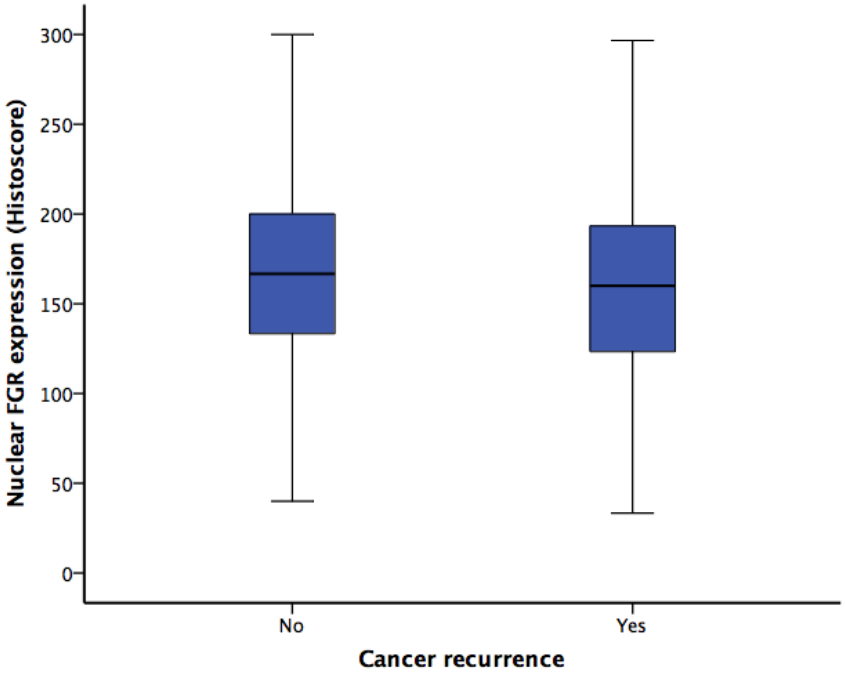


Supplementary figure 5.21: The distribution of cytoplasmic FGR measurements in patients with and without cancer recurrence in patients with stage II colorectal cancer (p=0.232)

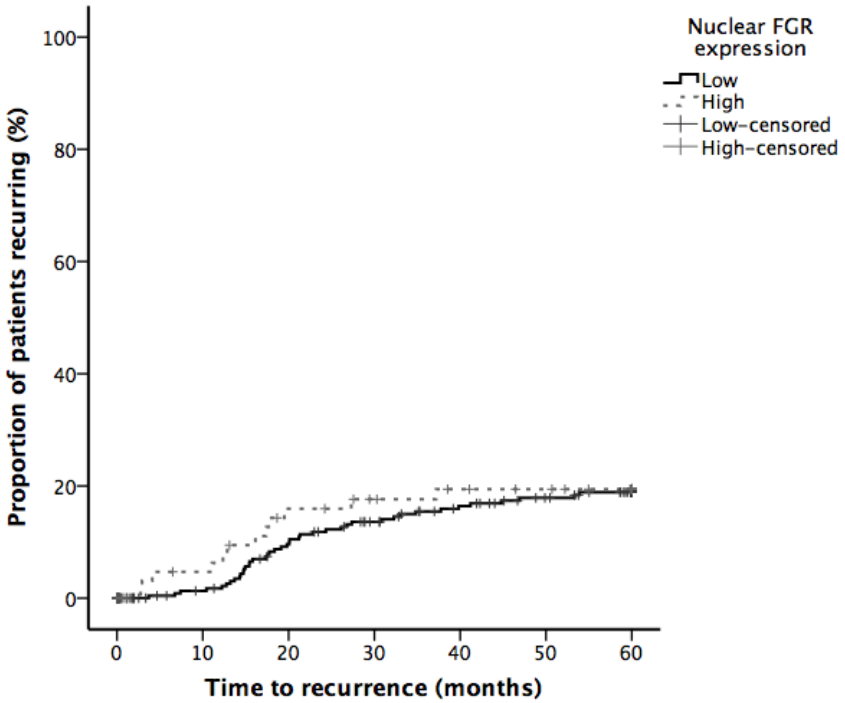


No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	245	213	190	178	164	152	139
High	82	74	66	58	55	54	52

Supplementary figure 5.22: The relationship between cytoplasmic FGR expression and recurrence-free survival in patients with stage II colorectal cancer

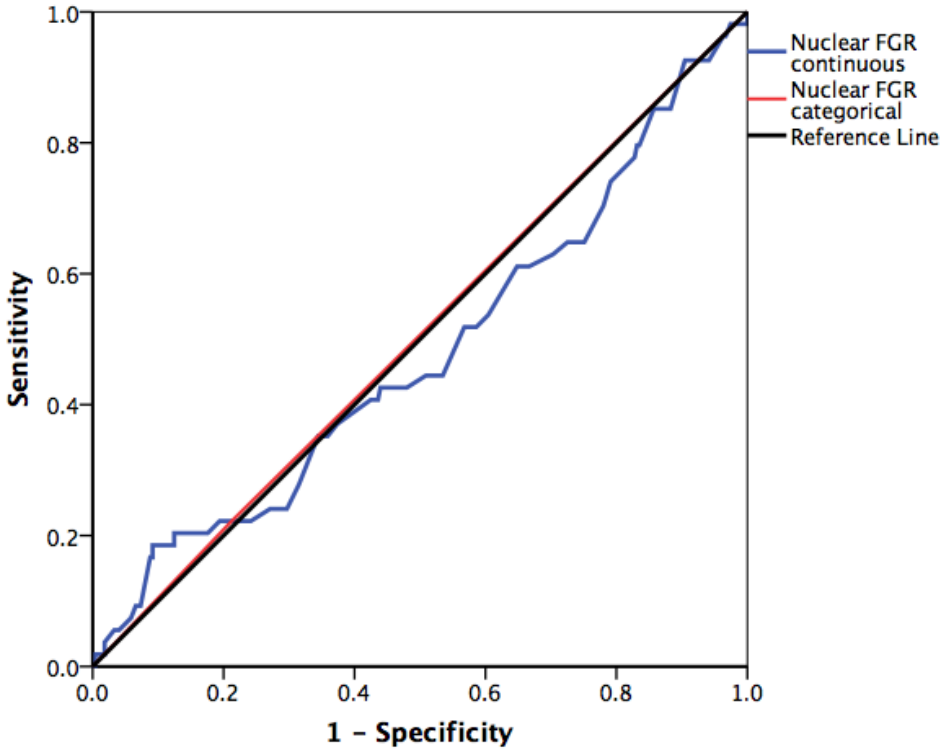


Supplementary figure 5.23: The distribution of nuclear FGR measurements in stage II colorectal cancer patients stratified by recurrence status (p=0.612)

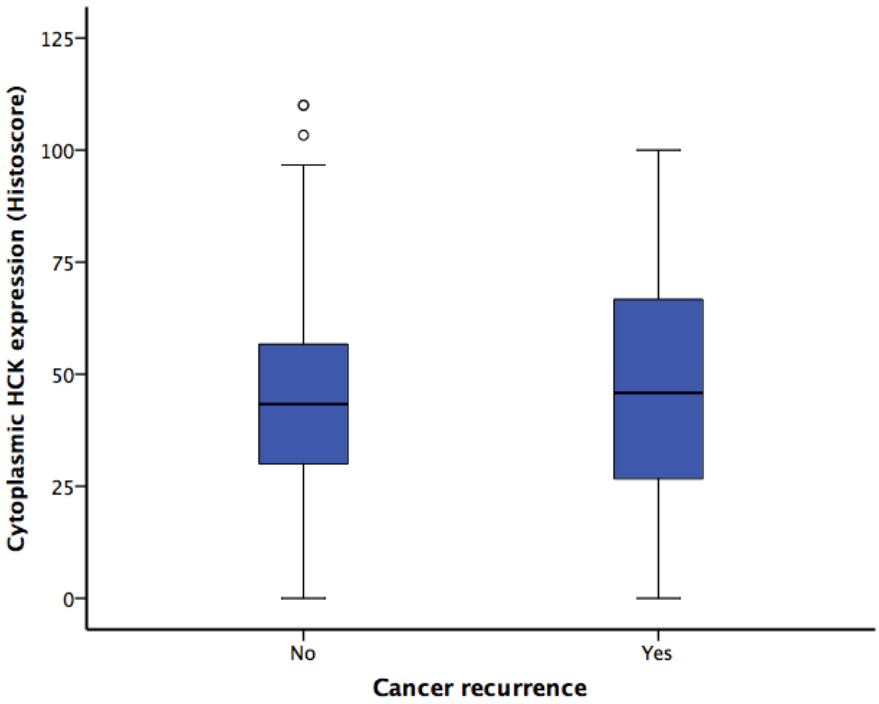


No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	257	227	205	189	175	164	153
Low	70	60	51	47	44	42	38

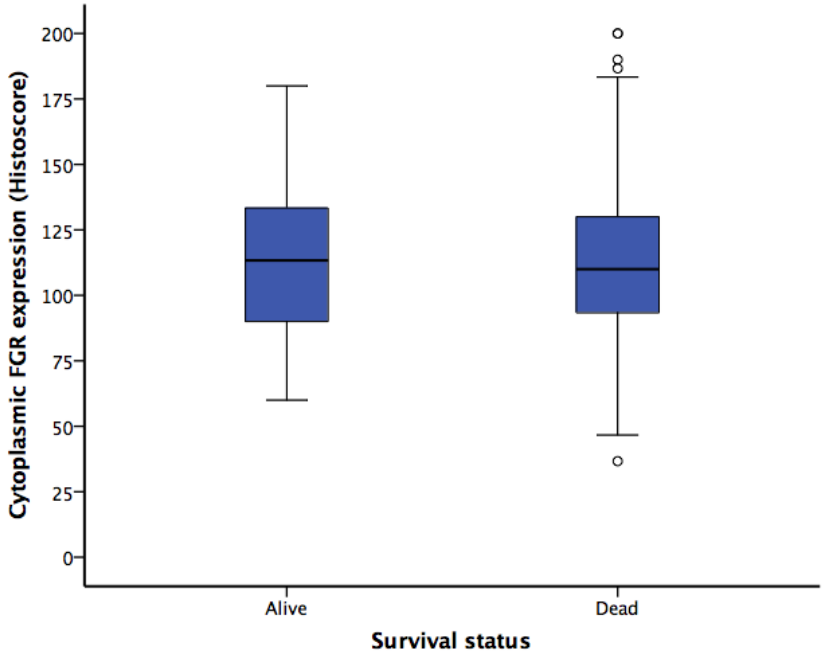
Supplementary figure 5.24: The relationship between nuclear FGR expression and recurrence-free survival in patients with stage II colorectal cancer



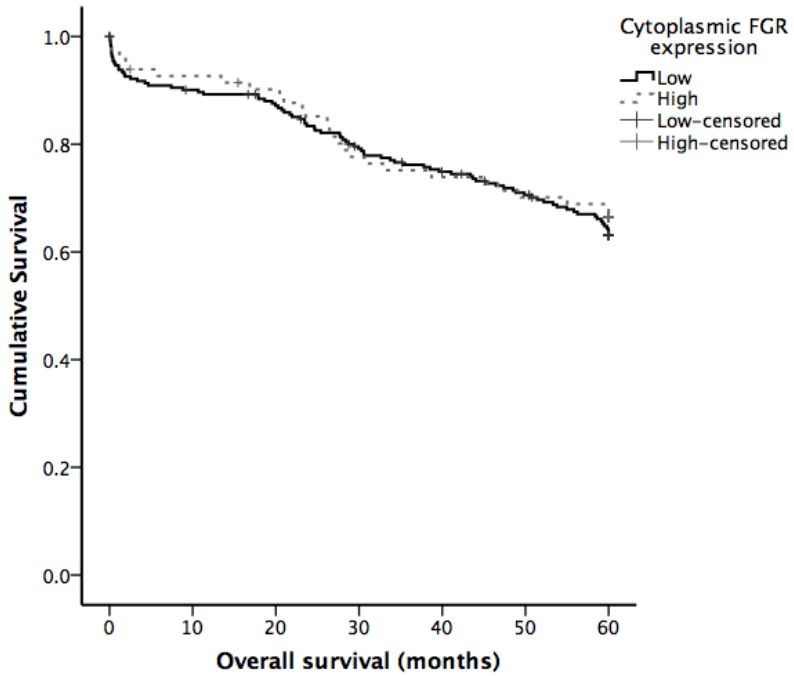
Supplementary figure 5.25: Predictive value of nuclear FGR in identifying patients with stage II colorectal cancer who will develop cancer recurrence



Supplementary figure 5.26: The distribution of cytoplasmic HCK measurements in patients with and without cancer recurrence in patients with stage II colorectal cancer (p=0.414)

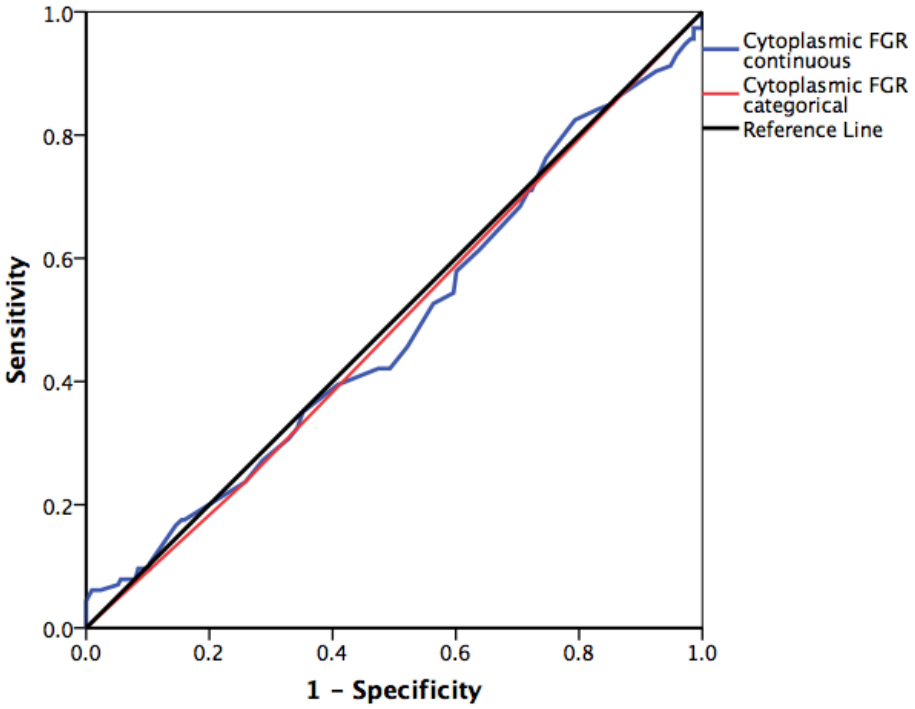


Supplementary figure 5.27: The distribution of cytoplasmic FGR measurements in patients stratified by survival status (p=0.730)

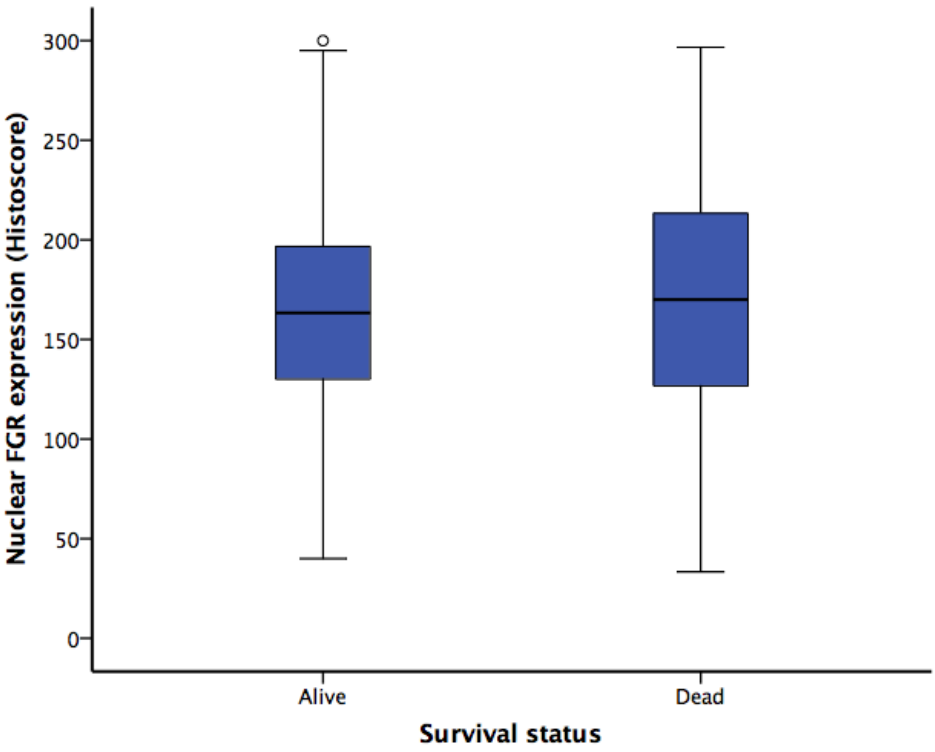


No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	245	217	208	187	174	162	144
High	82	75	72	62	59	56	55

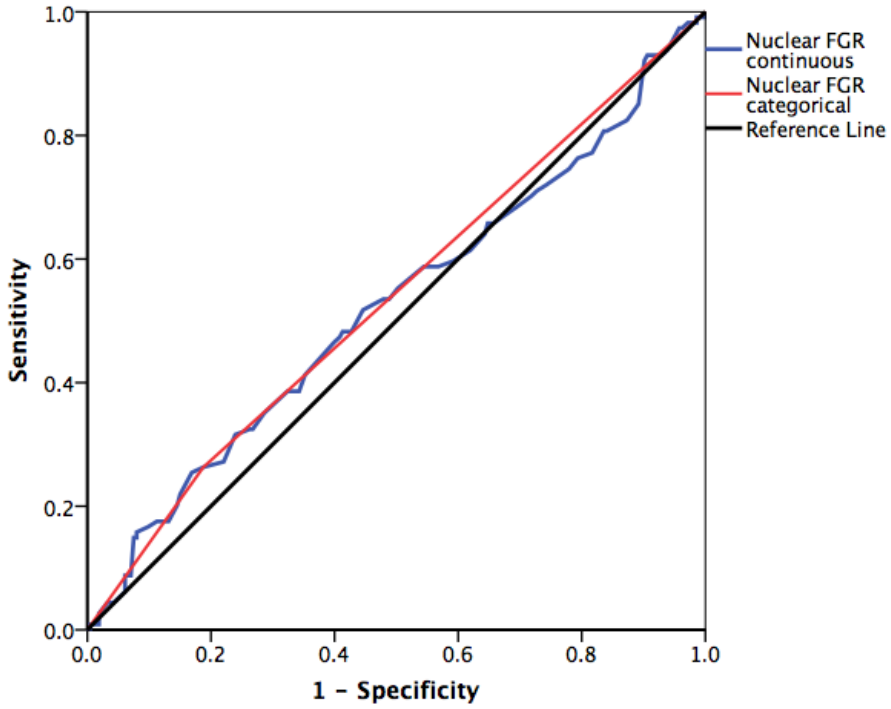
Supplementary figure 5.28: The relationship between cytoplasmic FGR expression and overall survival in patients with stage II colorectal cancer



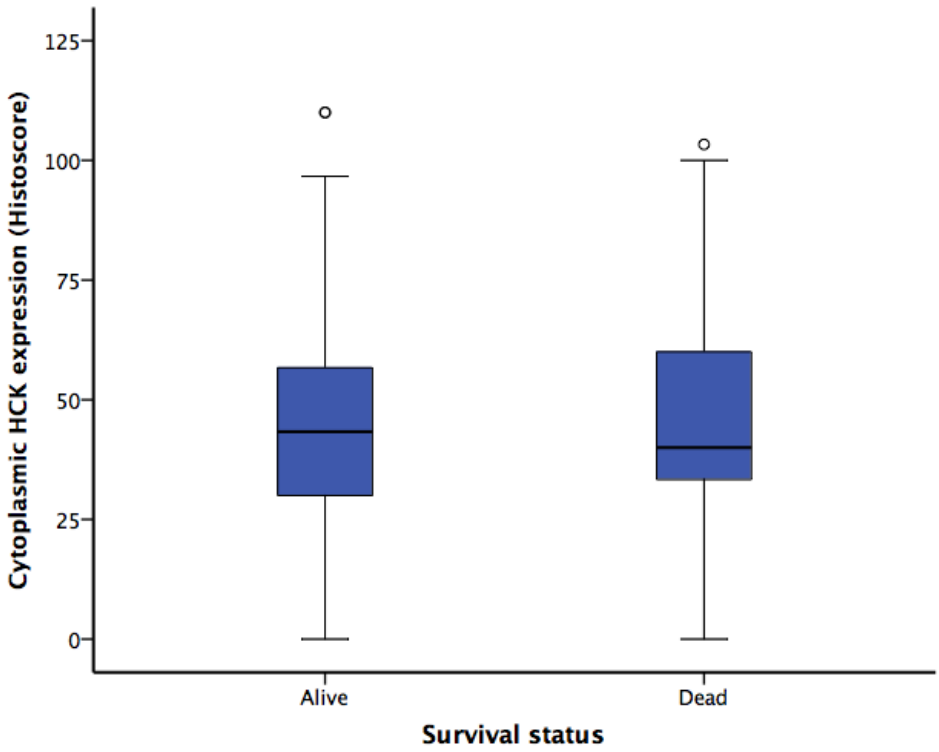
Supplementary figure 5.29: The predictive value of cytoplasmic FGR in identifying patients with stage II colorectal cancer who will die during follow-up



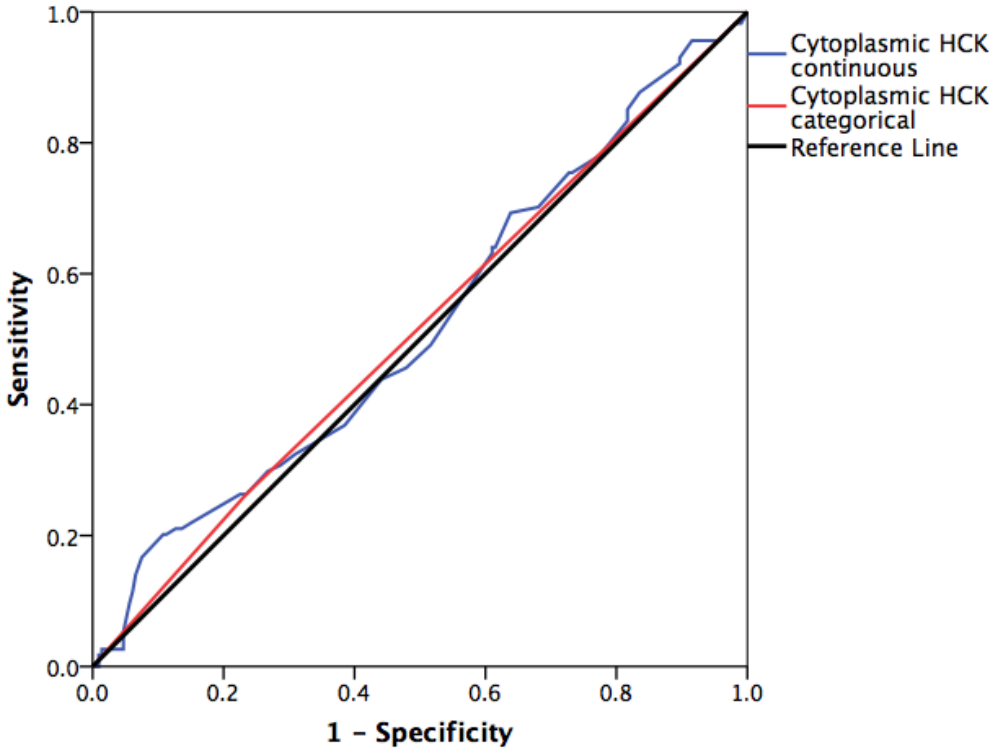
Supplementary figure 5.30: The distribution of nuclear FGR measurements in patients stratified by survival status (p=0.460)



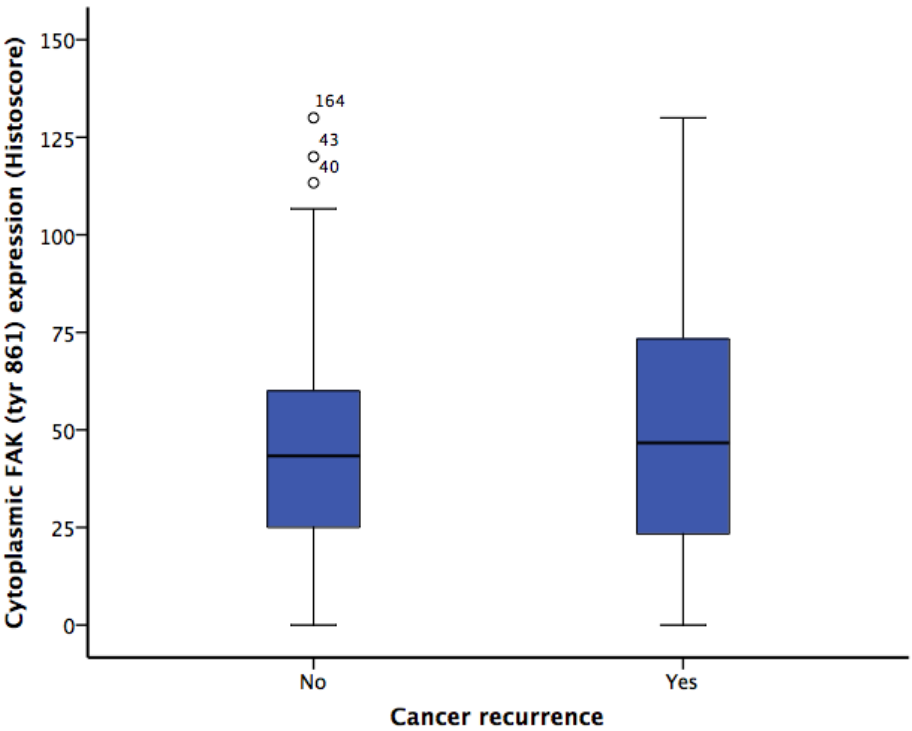
Supplementary figure 5.31: Predictive value of nuclear FGR in identifying patients with stage II colorectal cancer who will die during the follow-up



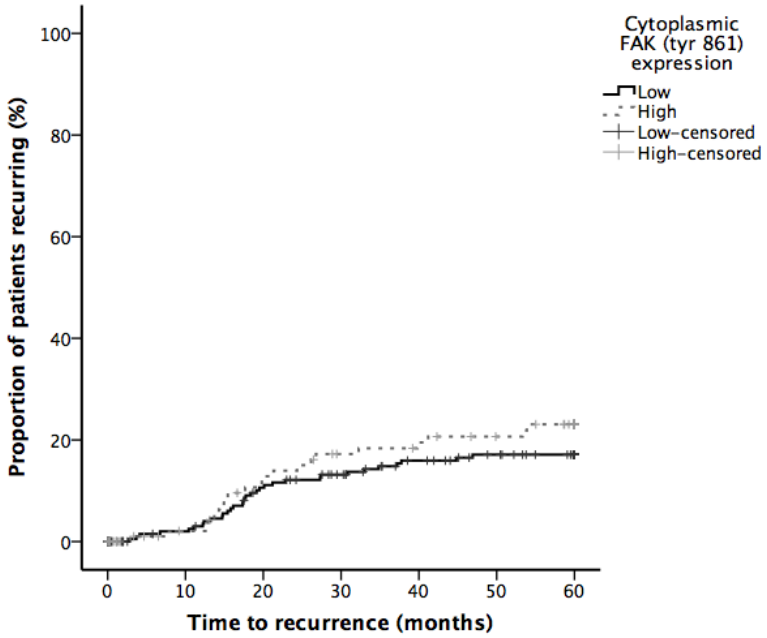
Supplementary figure 5.32: The distribution of cytoplasmic HCK measurements in patients stratified by survival status (p=0.532)



Supplementary figure 5.33: The predictive value of cytoplasmic HCK in identifying patients with stage II colorectal cancer who will die during follow-up

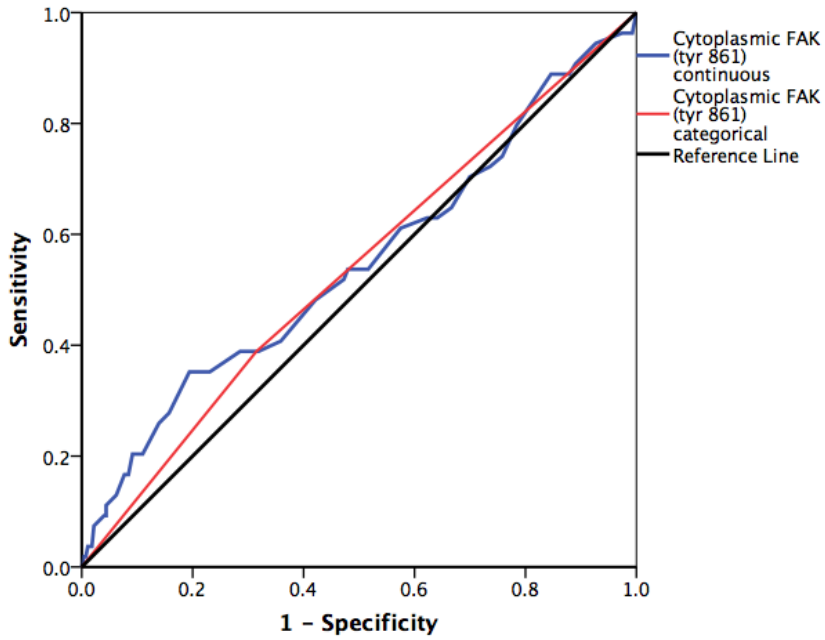


Supplementary figure 5.34: The distribution of cytoplasmic FAK (tyr 861) measurements in stage II colorectal cancer patients stratified by recurrence status (p=0.280)

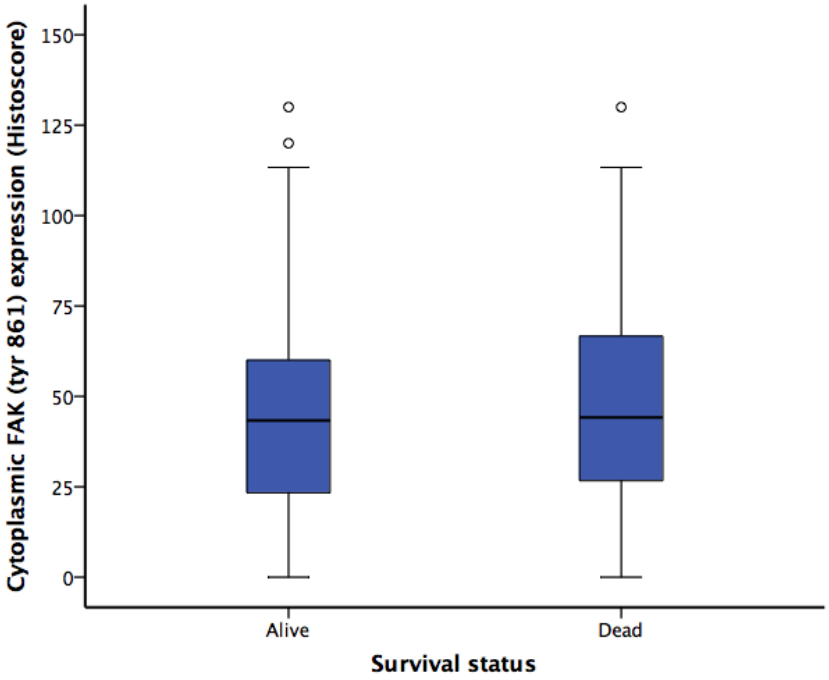


No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	220	195	175	163	149	140	131
Low	107	92	81	73	70	66	60

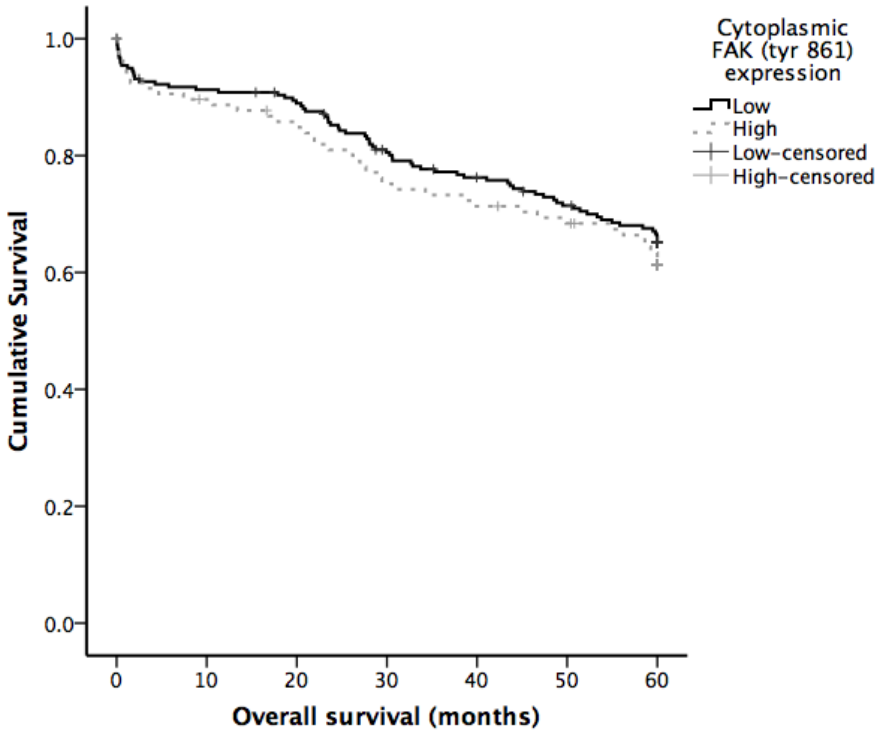
Supplementary figure 5.35: The relationship between cytoplasmic FAK (tyr 861) expression and recurrence-free survival in patients with stage II colorectal cancer



Supplementary figure 5.36 Predictive value of cytoplasmic FAK (tyr 861) in identifying patients with stage II colorectal cancer who will develop cancer recurrence

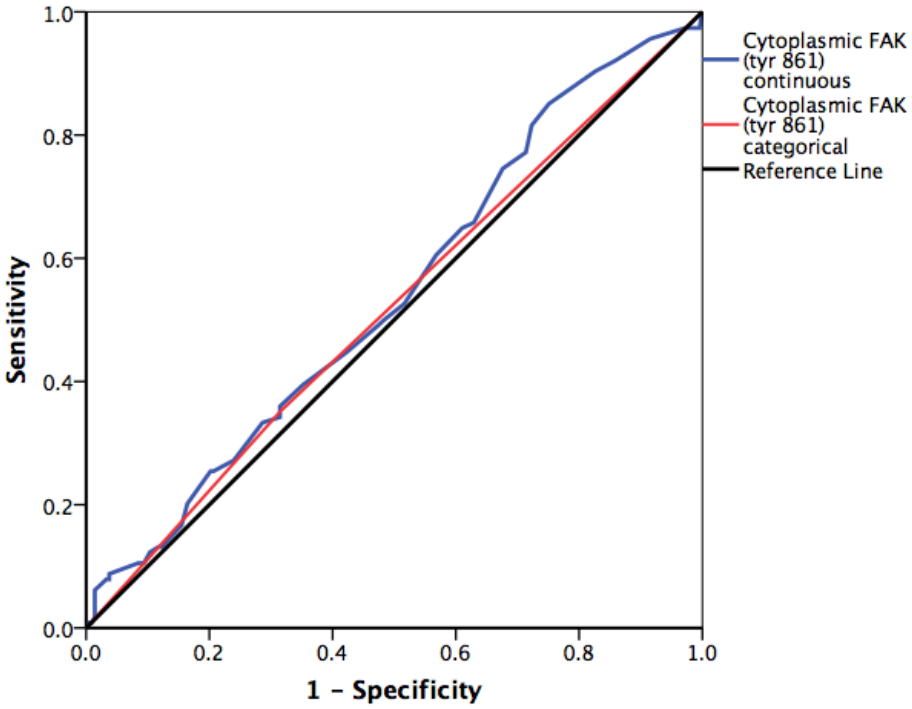


Supplementary figure 5.37: The distribution of cytoplasmic FAK (tyr 861) measurements in patients stratified by survival status (p=0.231)

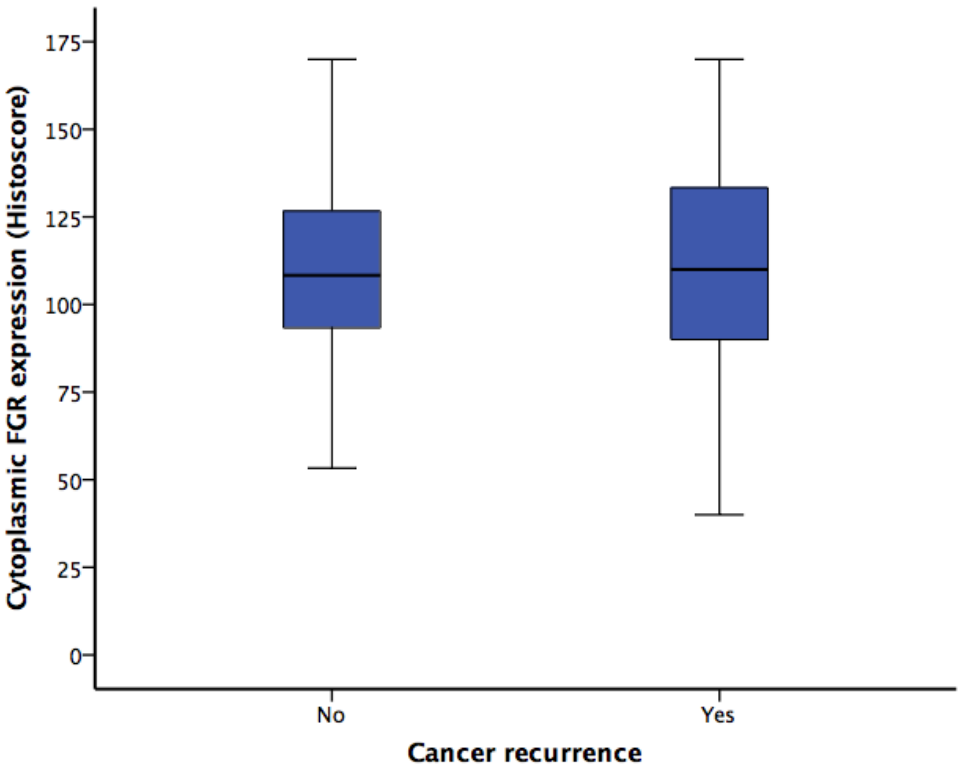


No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	220	198	191	170	159	148	136
Low	107	94	89	78	74	70	63

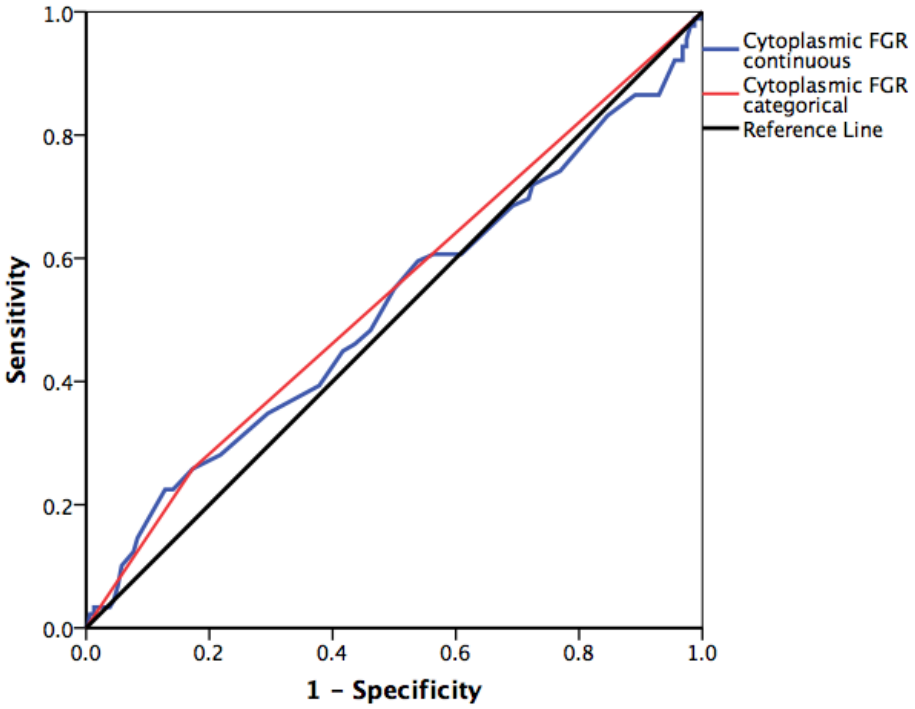
Supplementary figure 5.38: The relationship between cytoplasmic FAK (tyr 861) expression and overall survival in patients with stage II colorectal cancer



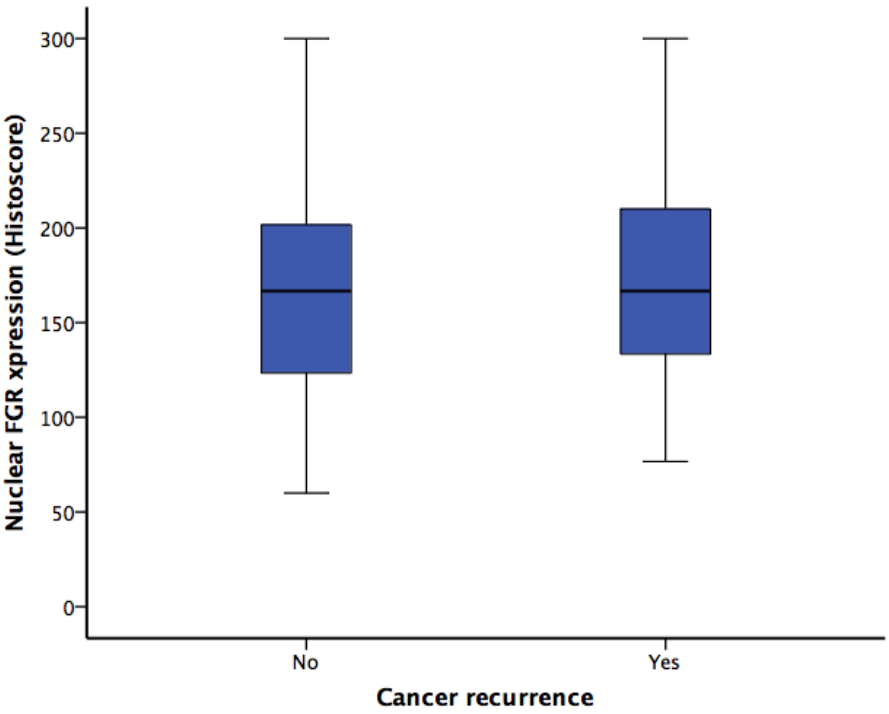
Supplementary figure 5.39: Predictive value of cytoplasmic FAK (tyr 861) in identifying patients with stage II colorectal cancer who will die during the follow-up



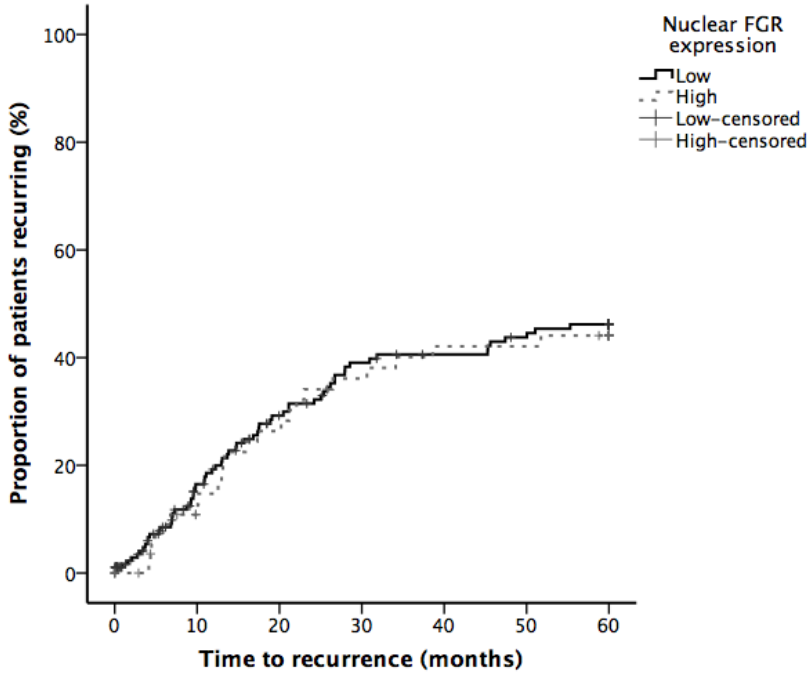
Supplementary figure 5.40: The distribution of cytoplasmic FGR measurements in patients with and without cancer recurrence in patients with stage III colorectal cancer (p=0.609)



Supplementary figure 5.41 The predictive value of cytoplasmic FGR in identifying patients with stage III colorectal cancer who will develop recurrence during the follow-up

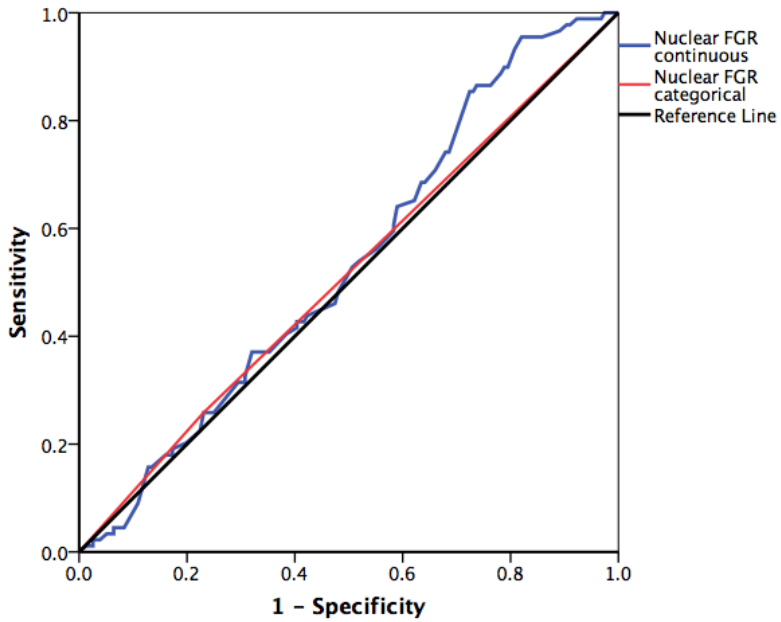


Supplementary figure 5.42: The distribution of nuclear FGR measurements in stage III colorectal cancer patients stratified by recurrence status (p=0.351)

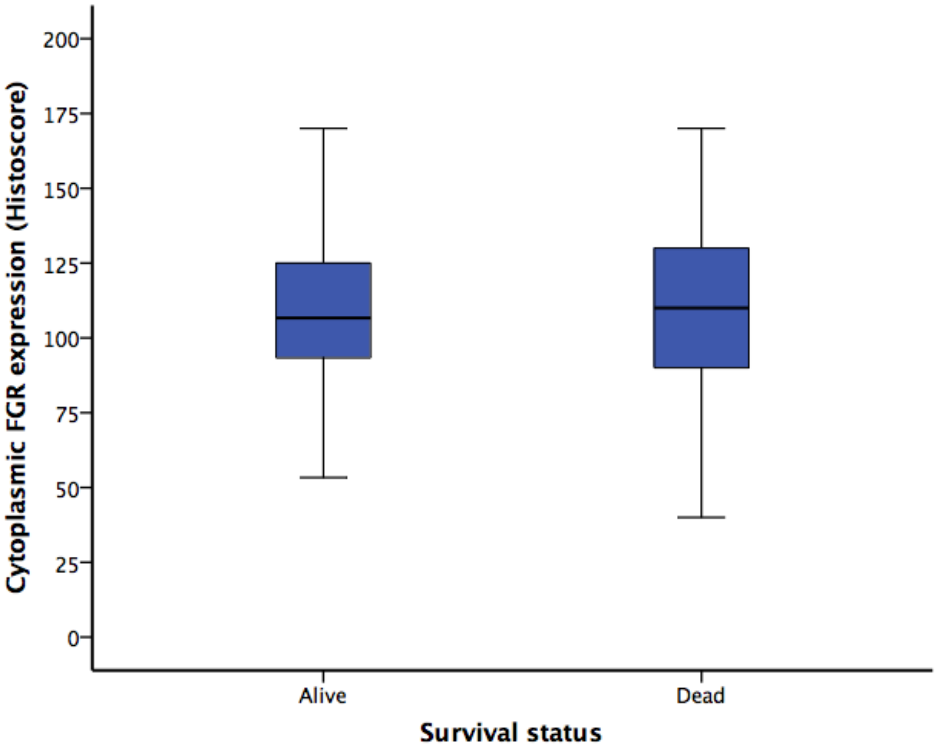


No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	186	122	95	80	75	70	67
Low	59	46	38	32	29	29	27

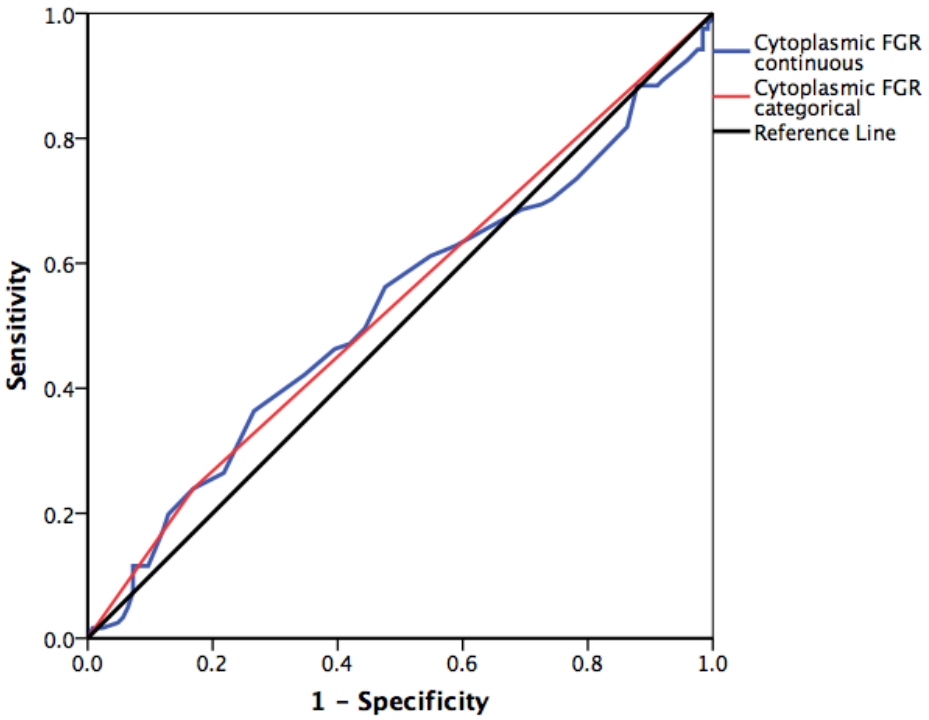
Supplementary figure 5.43: The relationship between nuclear FGR expression and recurrence-free survival in patients with stage III colorectal cancer



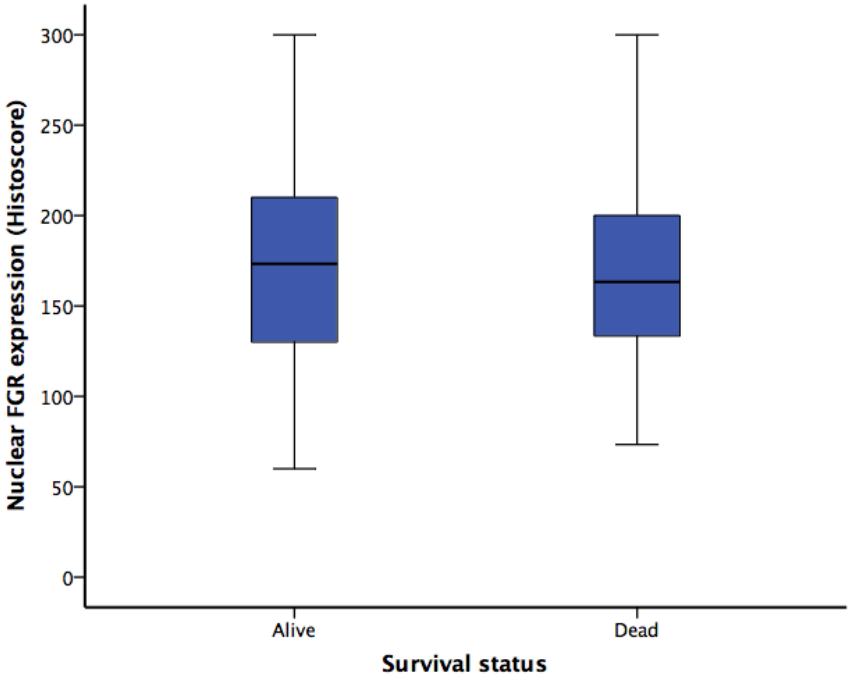
Supplementary figure 5.44 Predictive value of nuclear FGR in identifying patients with stage III colorectal cancer who will develop cancer recurrence



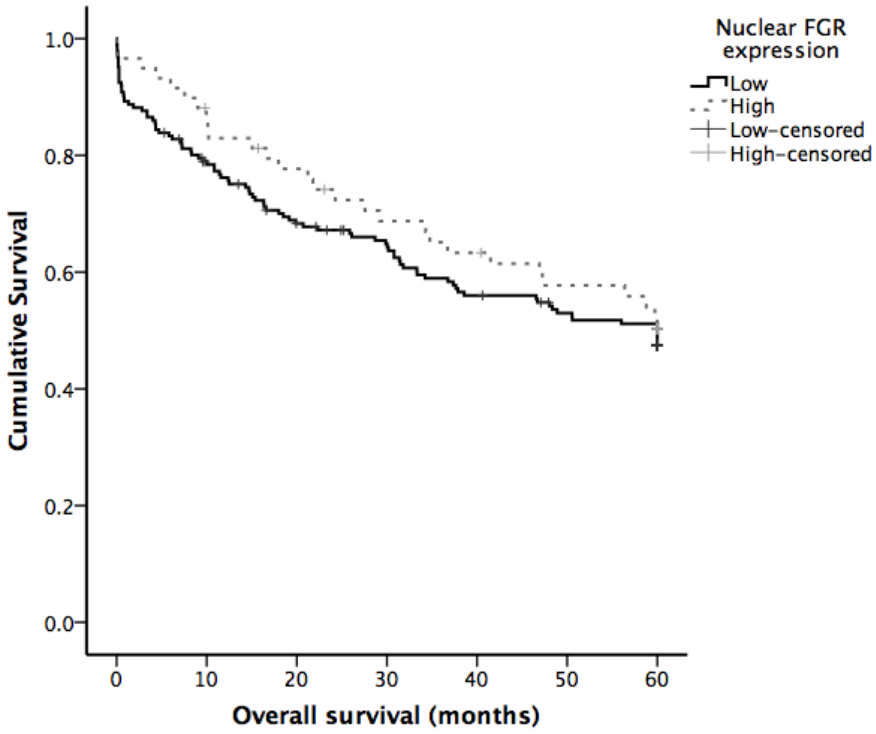
Supplementary figure 5.45: The distribution of cytoplasmic FGR measurements in patients stratified by survival status (p=0.494)



Supplementary figure 5.46: The predictive value of cytoplasmic FGR in identifying patients with stage III colorectal cancer who will die during follow-up

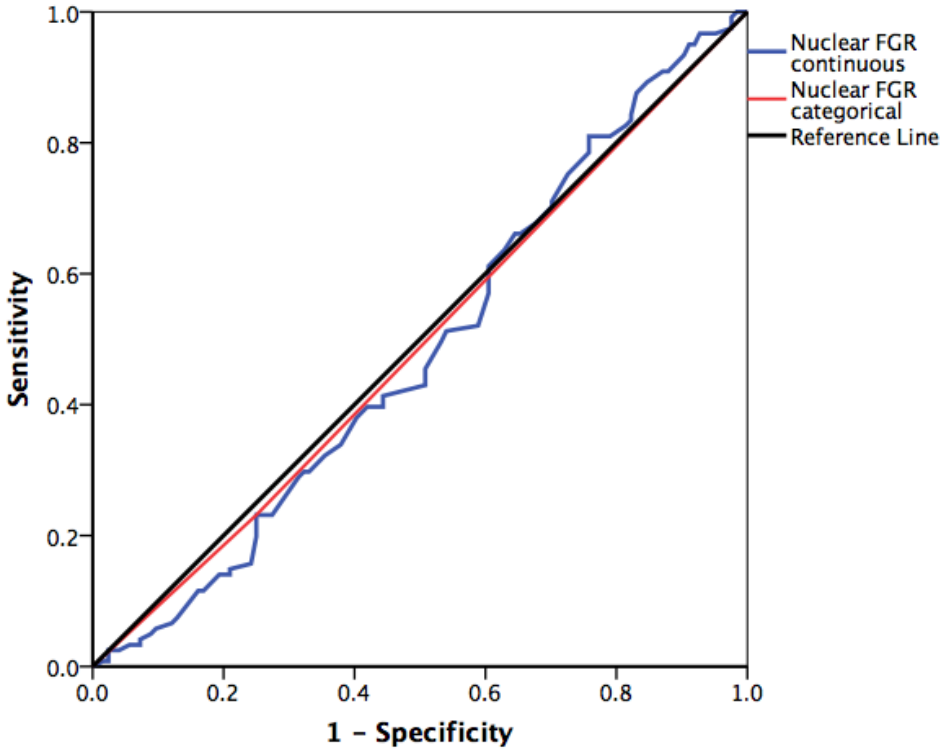


Supplementary figure 5.47: The distribution of nuclear FGR measurements in patients stratified by survival status (p=0.655)

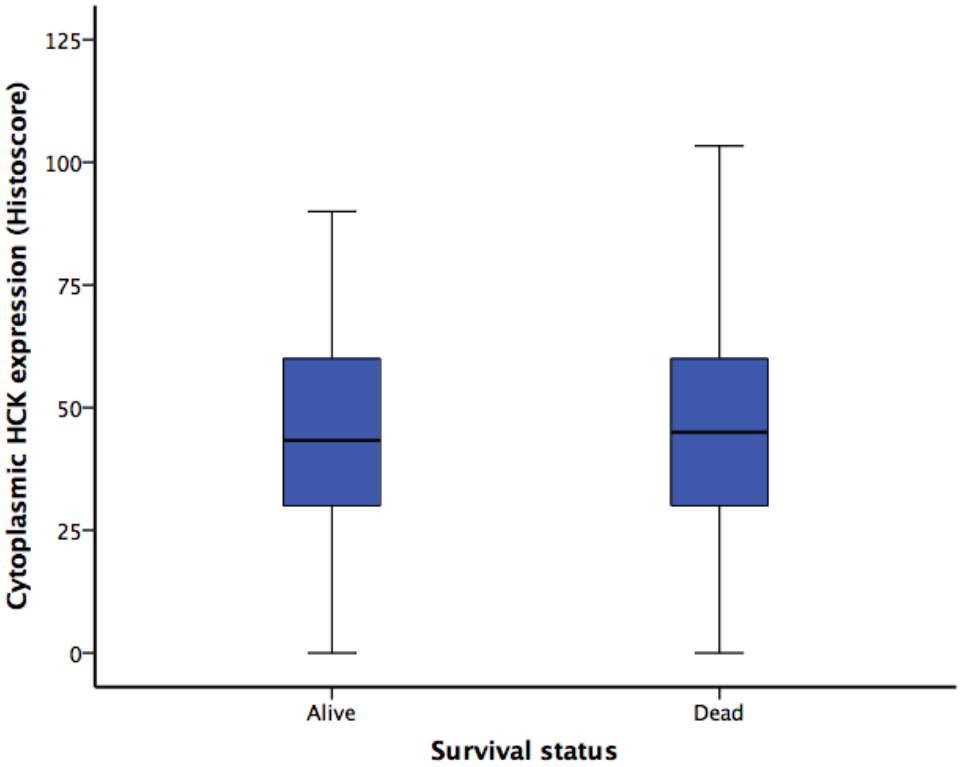


No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	186	142	120	110	96	87	84
Low	59	51	44	38	35	31	28

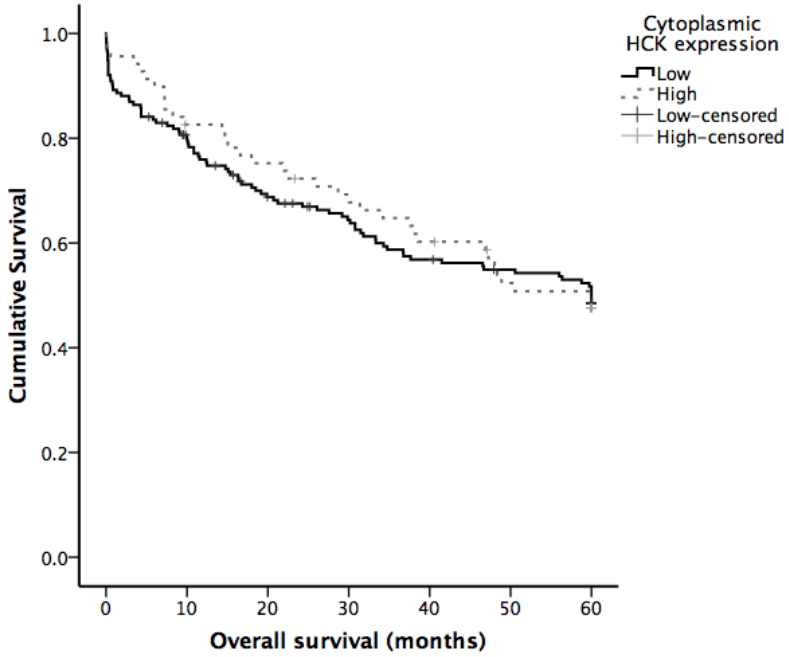
Supplementary figure 5.48: The relationship between nuclear FGR expression and overall survival in patients with stage III colorectal cancer



Supplementary figure 5.49: Predictive value of nuclear FGR in identifying patients with stage III colorectal cancer who will die during the follow-up

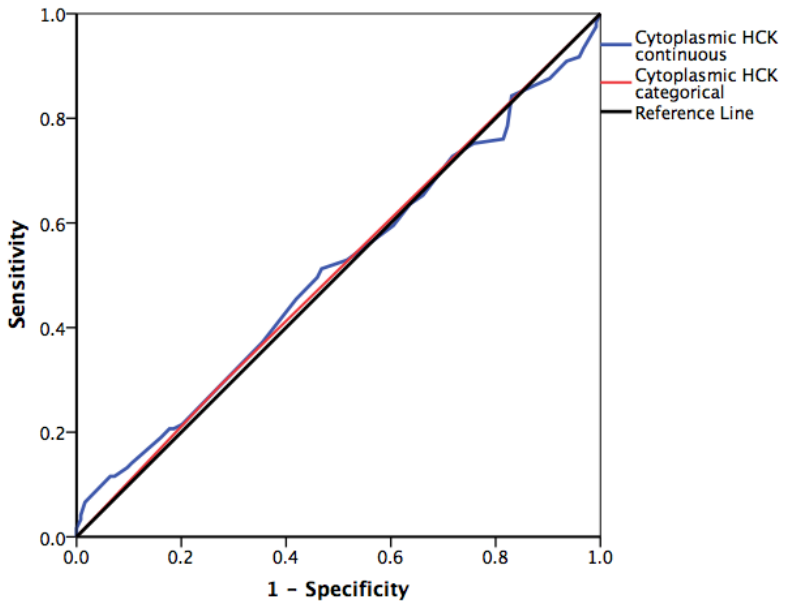


Supplementary figure 5.50: The distribution of cytoplasmic HCK measurements in patients stratified by survival status (p=0.805)

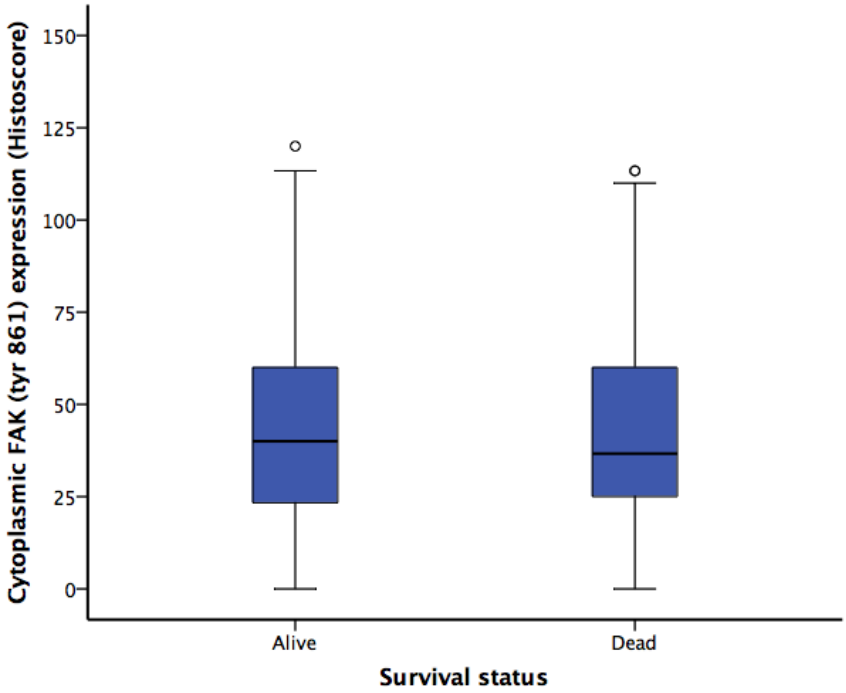


No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	176	136	113	102	90	85	80
High	69	56	51	46	40	33	32

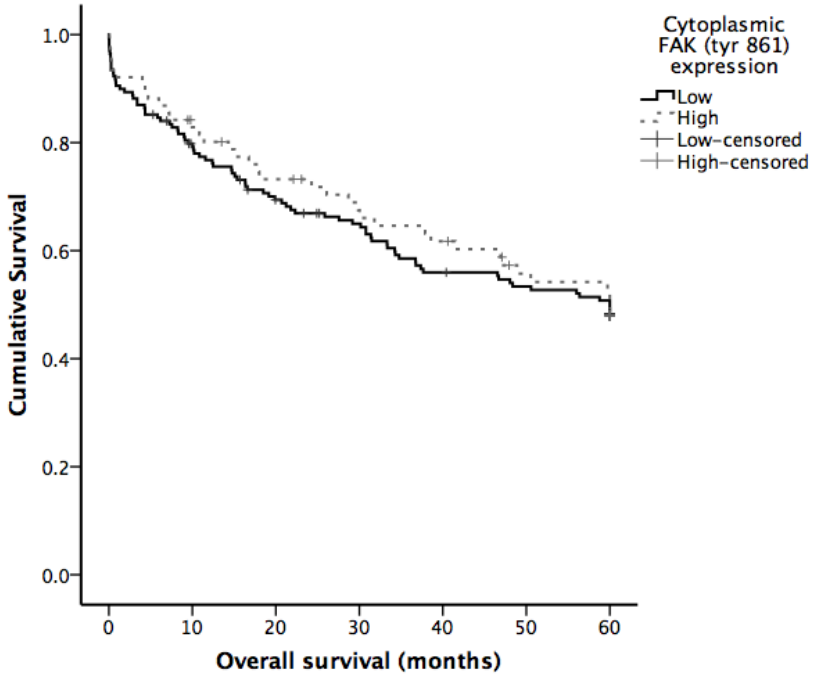
Supplementary figure 5.51: The relationship between cytoplasmic HCK expression and overall survival in patients with stage III colorectal cancer



Supplementary figure 5.52: The predictive value of cytoplasmic HCK in identifying patients with stage III colorectal cancer who will die during follow-up

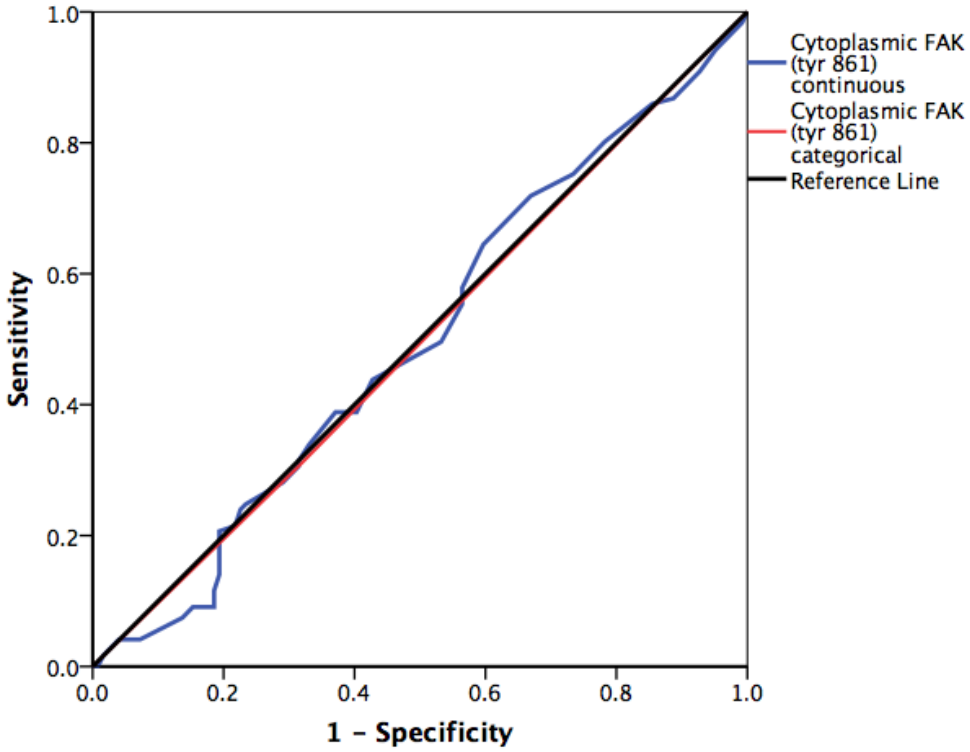


Supplementary figure 5.53: The distribution of cytoplasmic FAK (tyr 861) measurements in patients stratified by survival status (p=0.953)

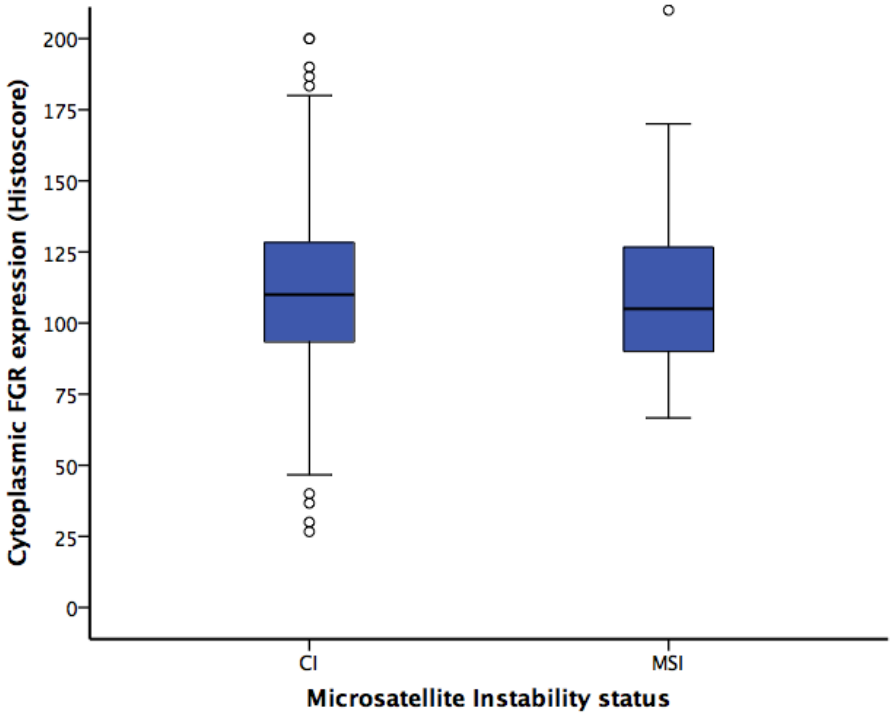


No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	169	131	111	101	87	82	78
Low	76	61	53	47	43	36	34

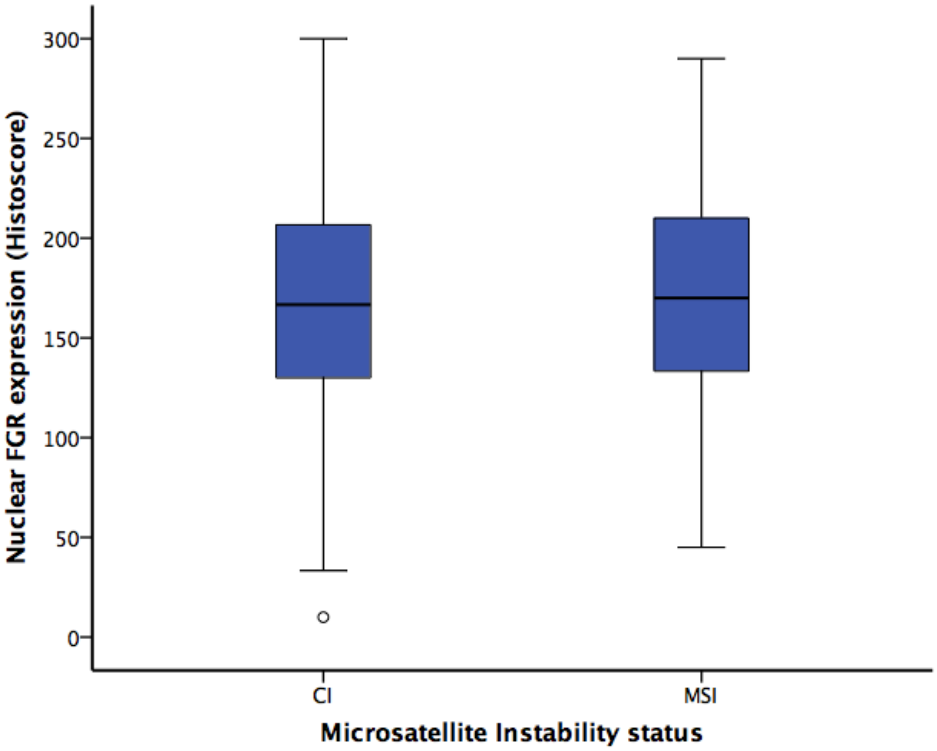
Supplementary figure 5.54: The relationship between cytoplasmic FAK (tyr 861) expression and overall survival in patients with stage III colorectal cancer



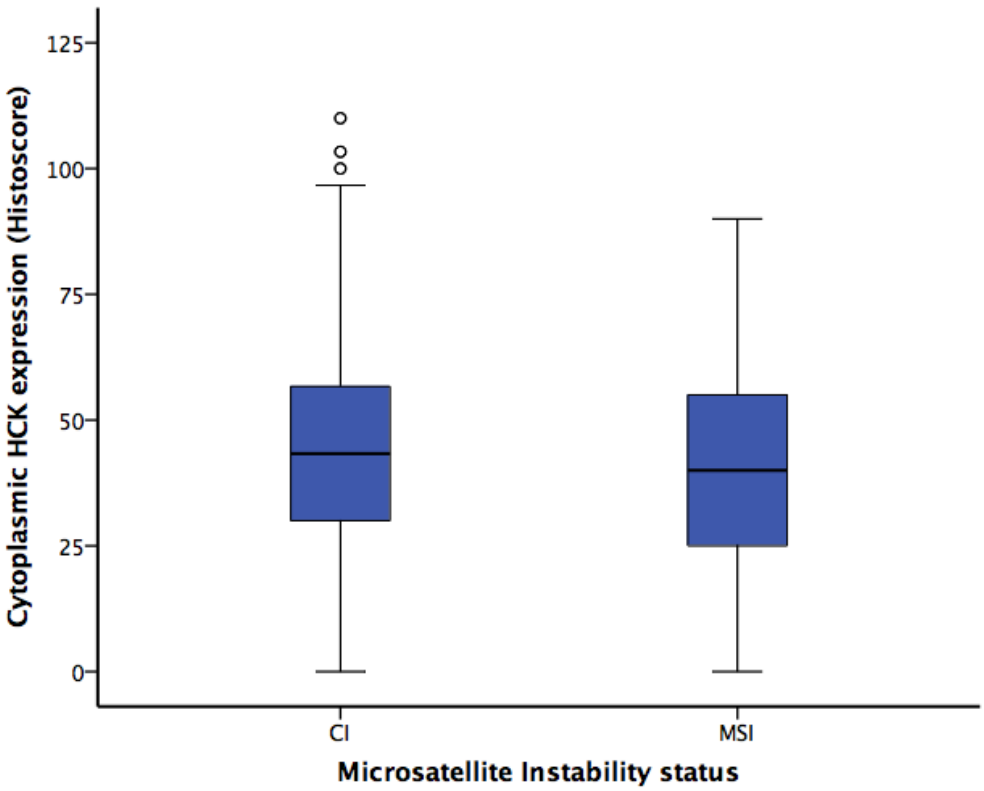
Supplementary figure 5.55: Predictive value of cytoplasmic FAK (tyr 861) in identifying patients with stage III colorectal cancer who will die during the follow-up



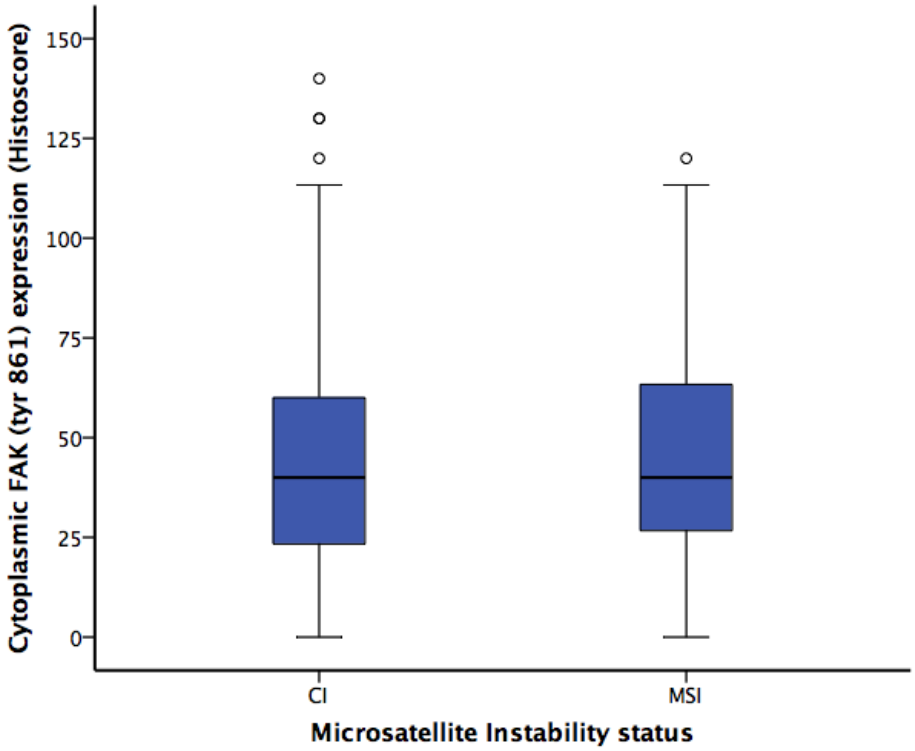
Supplementary figure 5.56: Distribution of measurements of cytoplasmic FGR expression in patients with CI and MSI colorectal cancer.



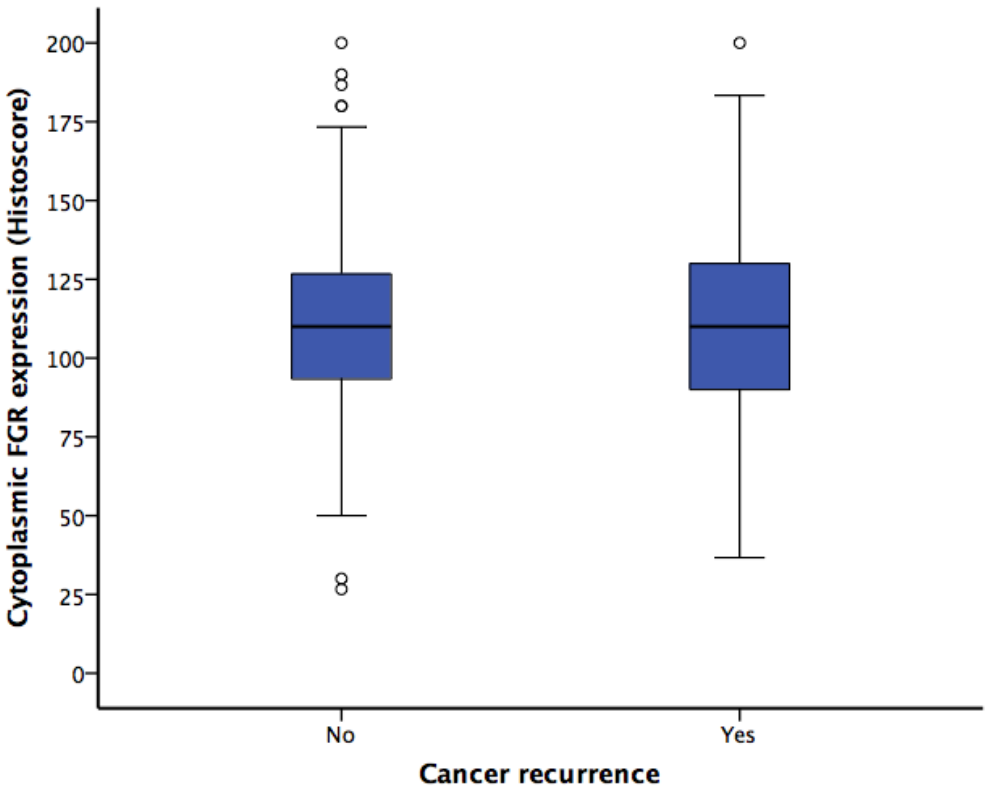
Supplementary figure 5.57: Distribution of measurements of nuclear FGR in patients with CI and MSI colorectal cancer.



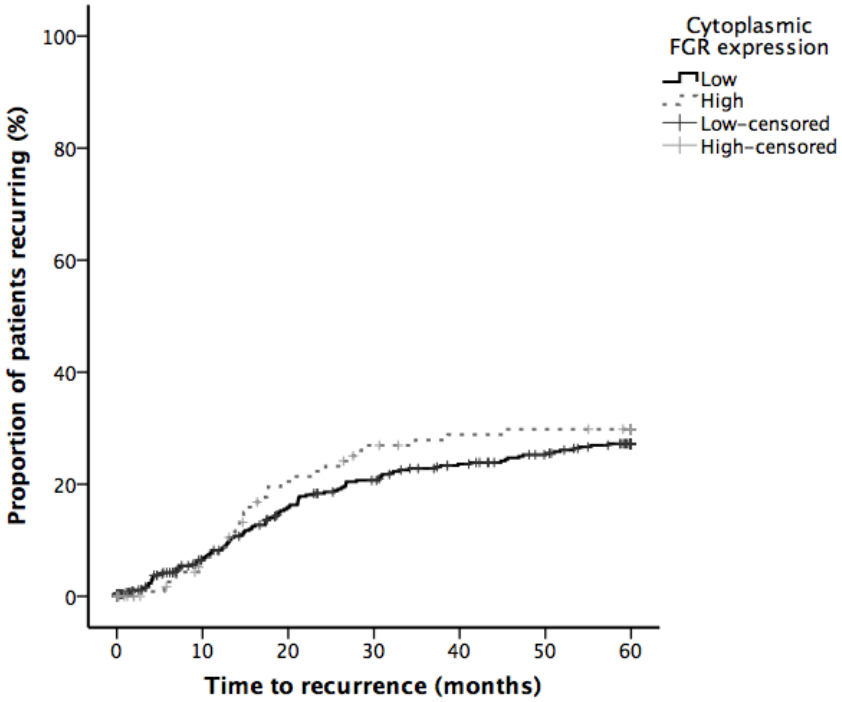
Supplementary figure 5.58: Distribution of measurements of cytoplasmic HCK in patients with CI and MSI colorectal cancer.



Supplementary figure 5.59: Distribution of measurements of cytoplasmic FAK (tyr 861) expression in patients with CI and MSI colorectal cancer

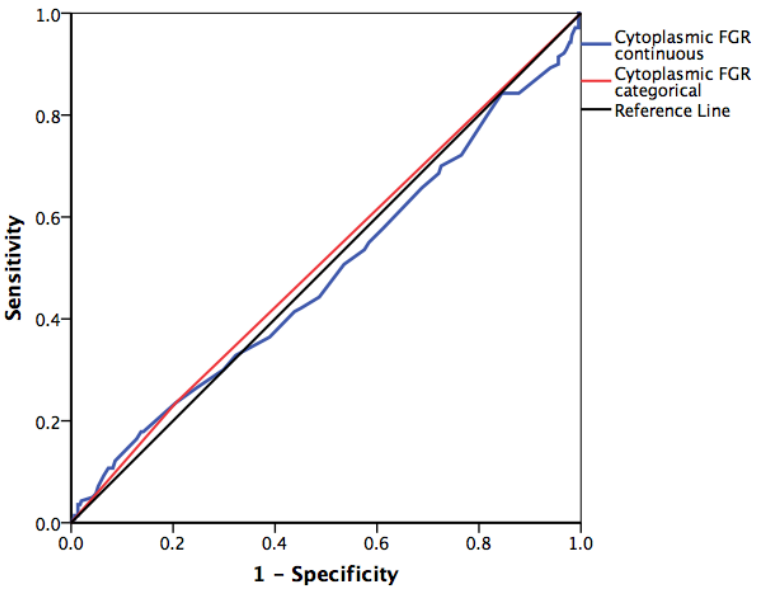


Supplementary figure 5.60: The distribution of cytoplasmic FGR measurements in patients with and without cancer recurrence in patients with CI colorectal cancer (p=0.646)

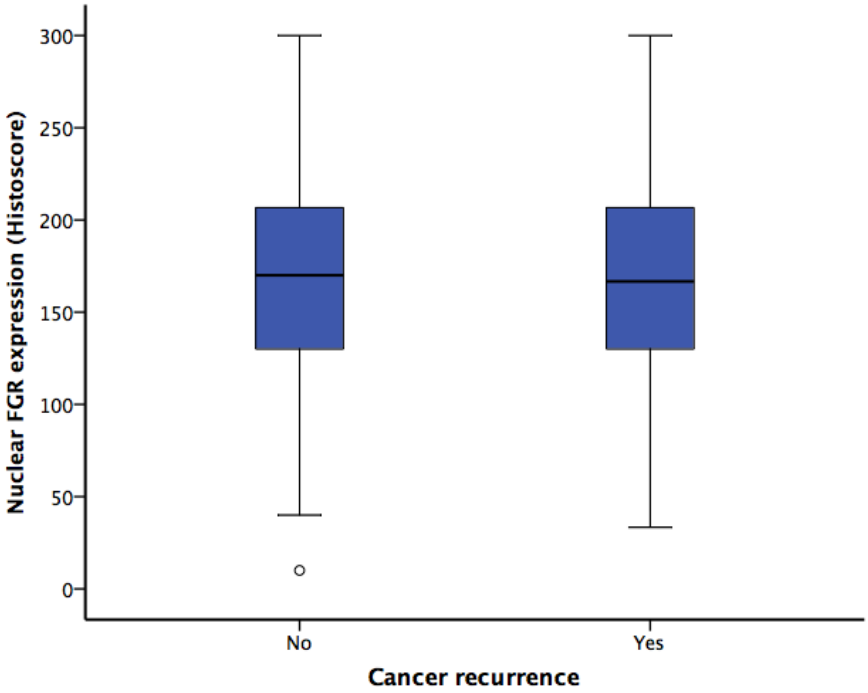


No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	466	374	328	304	283	268	246
High	126	105	87	78	74	73	71

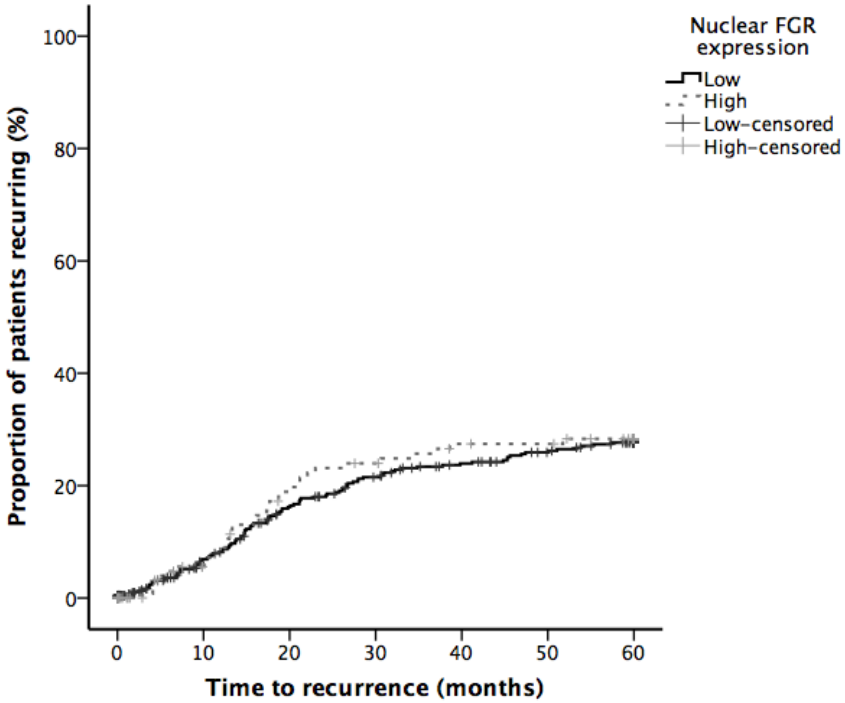
Supplementary figure 5.61: The relationship between cytoplasmic FGR expression and recurrence free survival in patients with CI colorectal cancer



Supplementary figure 5.62: The predictive value of cytoplasmic FGR in identifying patients with CI colorectal cancer who will develop recurrence during the follow-up

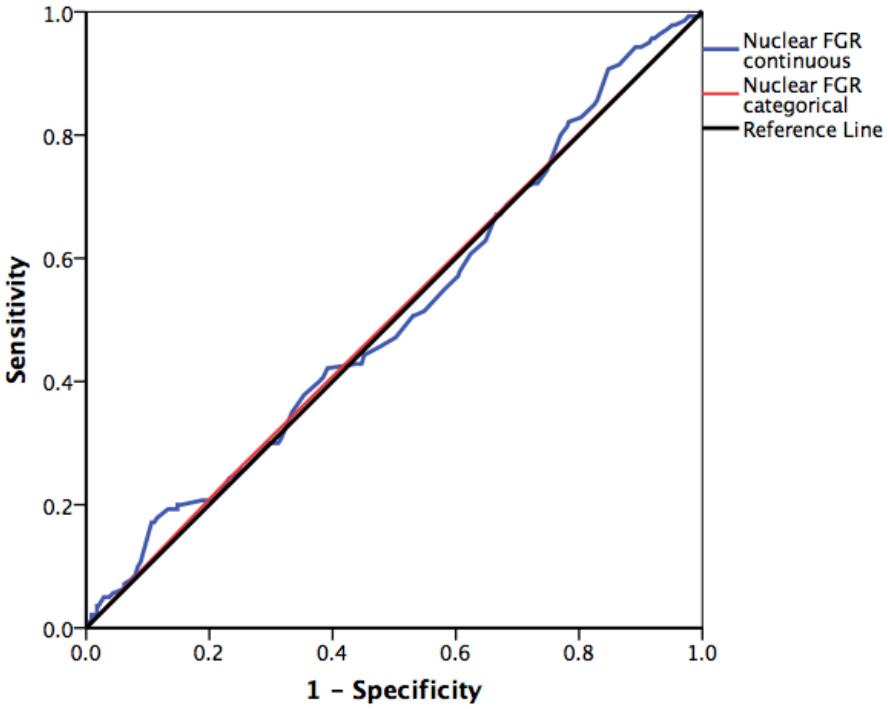


Supplementary figure 5.63: The distribution of nuclear FGR measurements in stage CI colorectal cancer patients stratified by recurrence status (p=0.715)

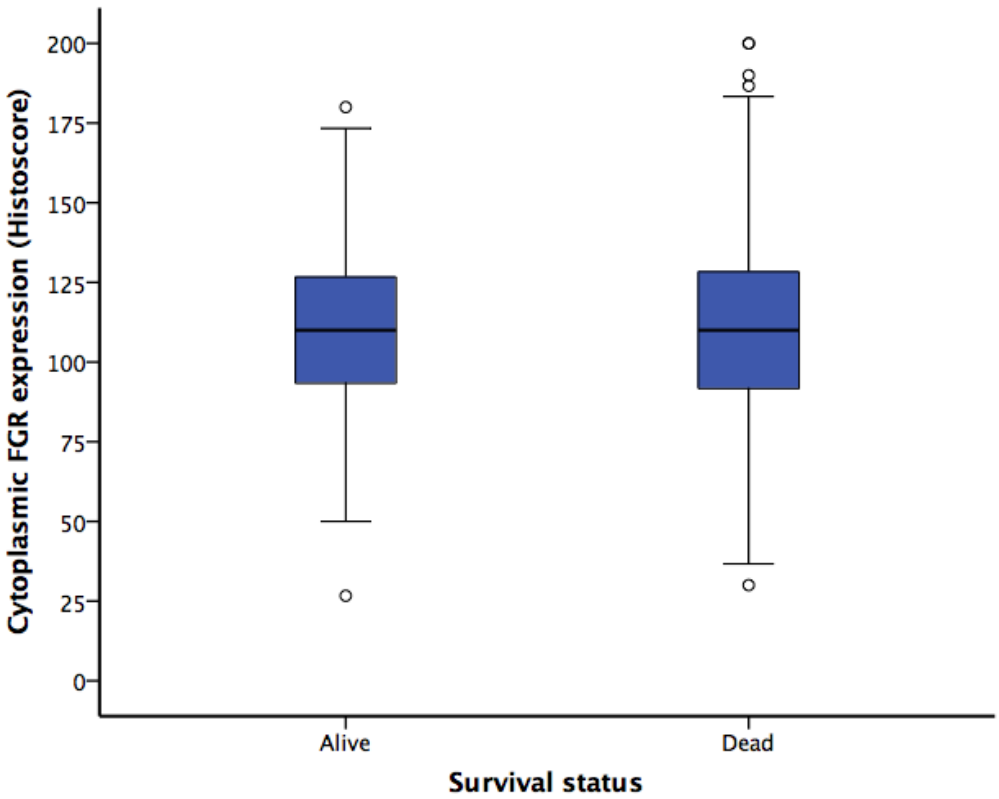


No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	453	365	319	293	274	259	242
Low	139	114	96	82	83	82	75

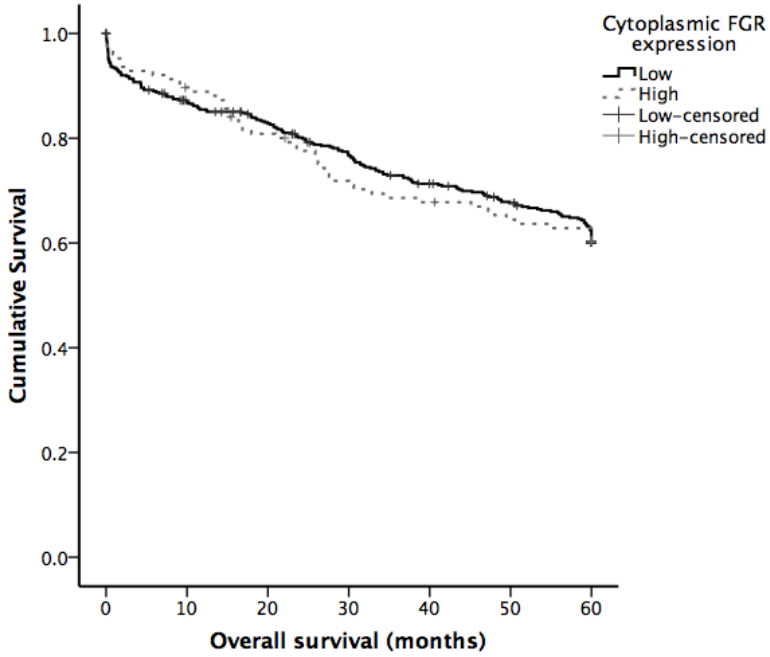
Supplementary figure 5.64: The relationship between nuclear FGR expression and recurrence-free survival in patients with CI colorectal cancer



Supplementary figure 5.65 Predictive value of nuclear FGR in identifying patients with CI colorectal cancer who will develop cancer recurrence

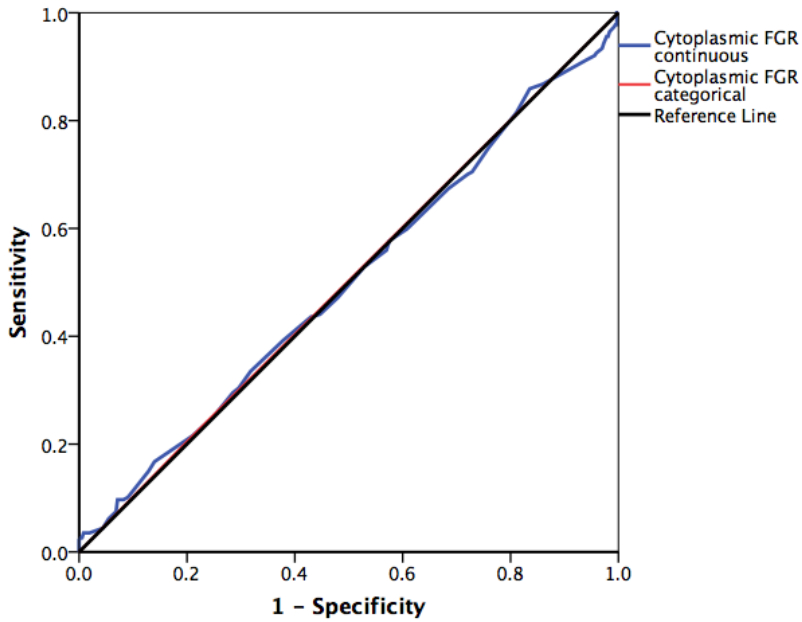


Supplementary figure 5.66: The distribution of cytoplasmic FGR measurements in patients stratified by survival status (p=0.978)

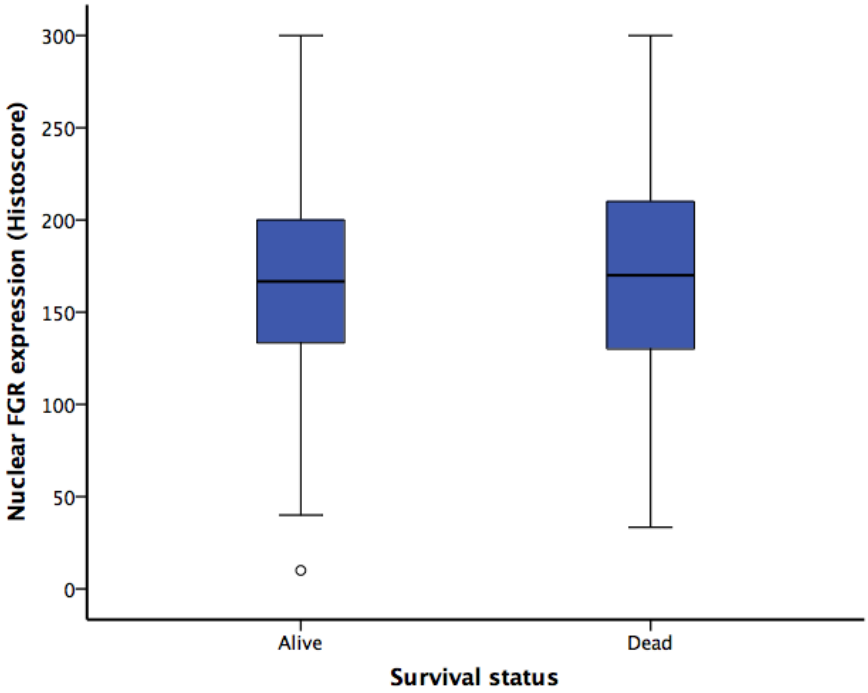


No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	466	396	371	339	312	292	267
High	126	112	100	87	83	78	76

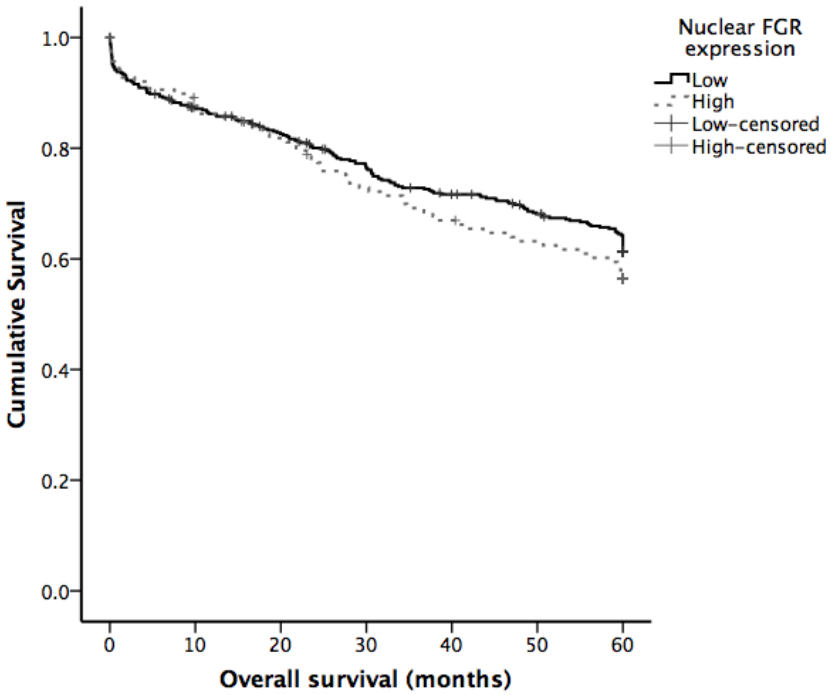
Supplementary figure 5.67: The relationship between cytoplasmic FGR expression and overall survival in patients with CI colorectal cancer



Supplementary figure 5.68: Predictive value of cytoplasmic FGR in identifying patients with CI colorectal cancer who will die during follow-up

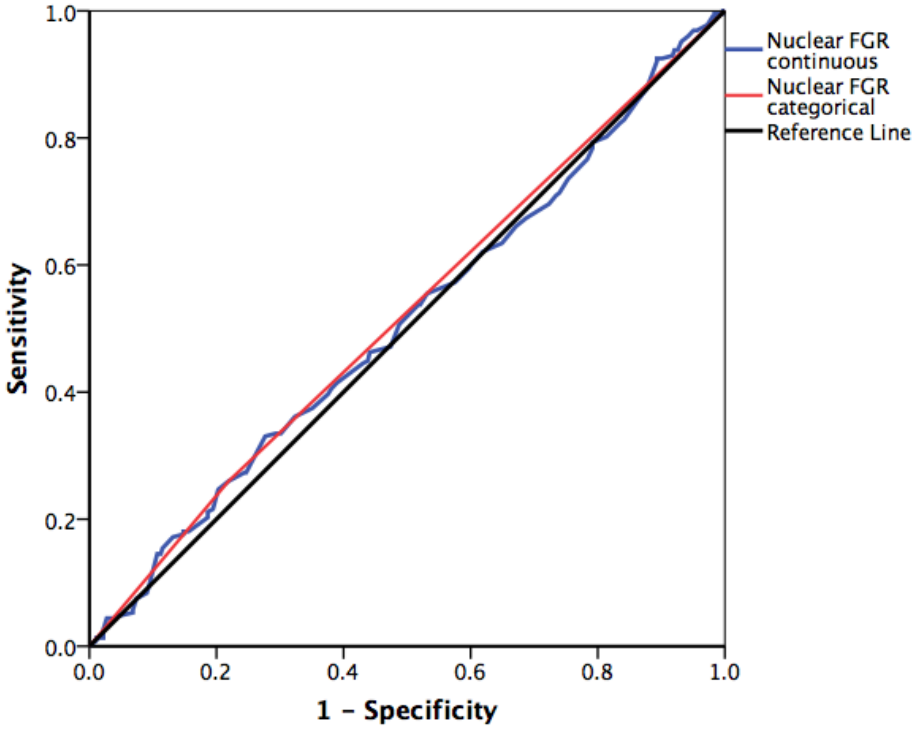


Supplementary figure 5.69: The distribution of nuclear FGR measurements in patients stratified by survival status (p=0.670)

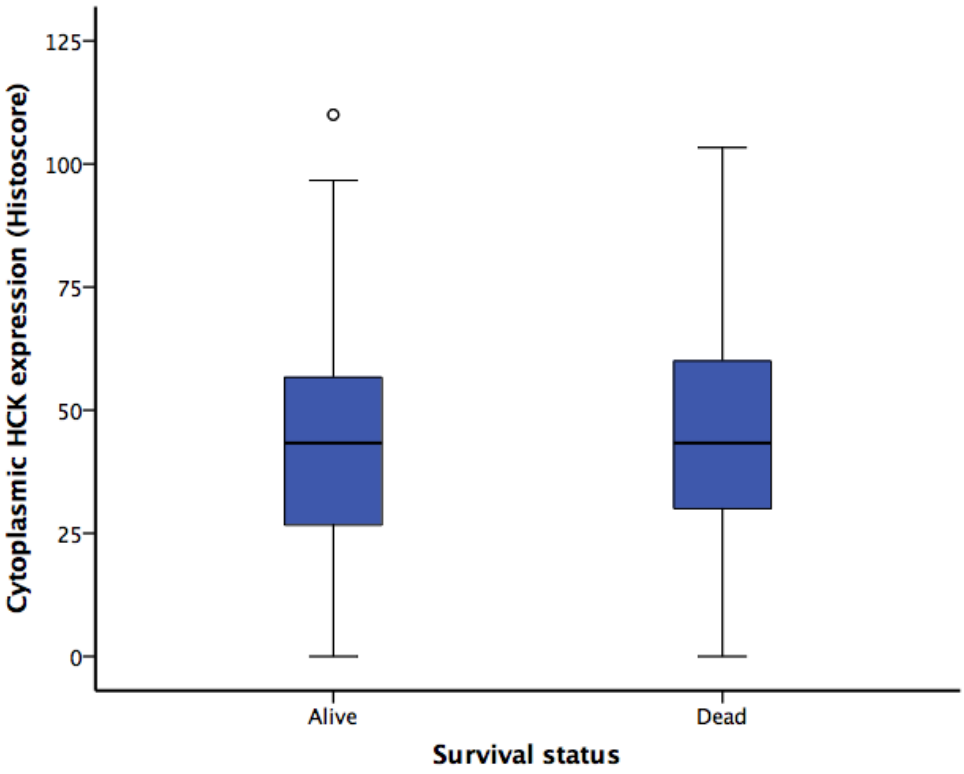


No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	453	385	360	328	305	286	267
Low	139	121	111	98	90	84	76

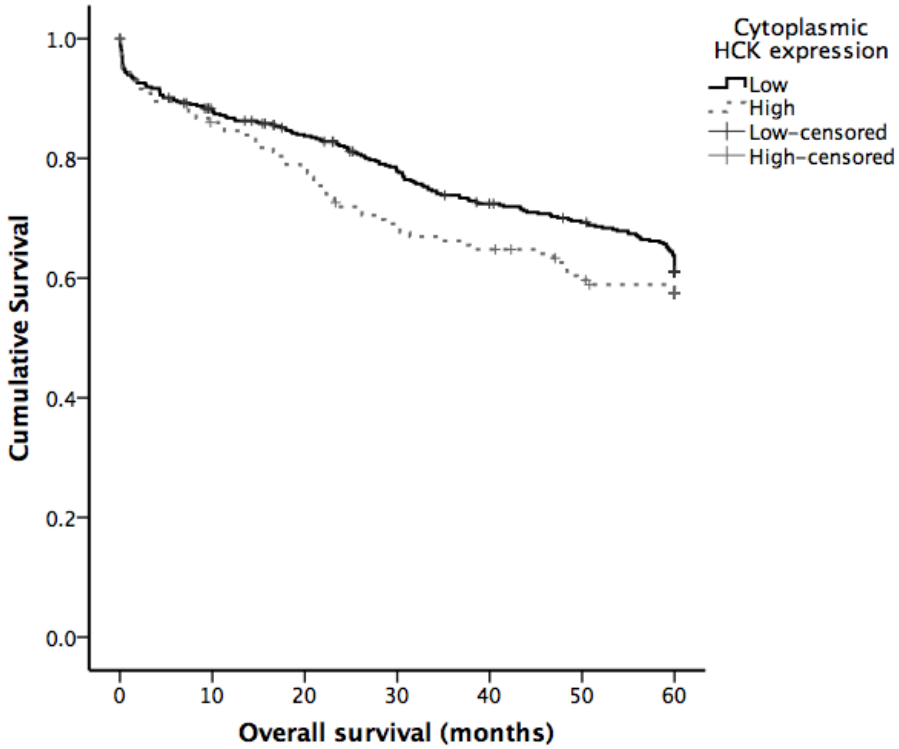
Supplementary figure 5.70: The relationship between nuclear FGR expression and overall survival in patients with CI colorectal cancer



Supplementary figure 5.71 Predictive value of nuclear FGR in identifying patients with CI colorectal cancer who will die during the follow-up

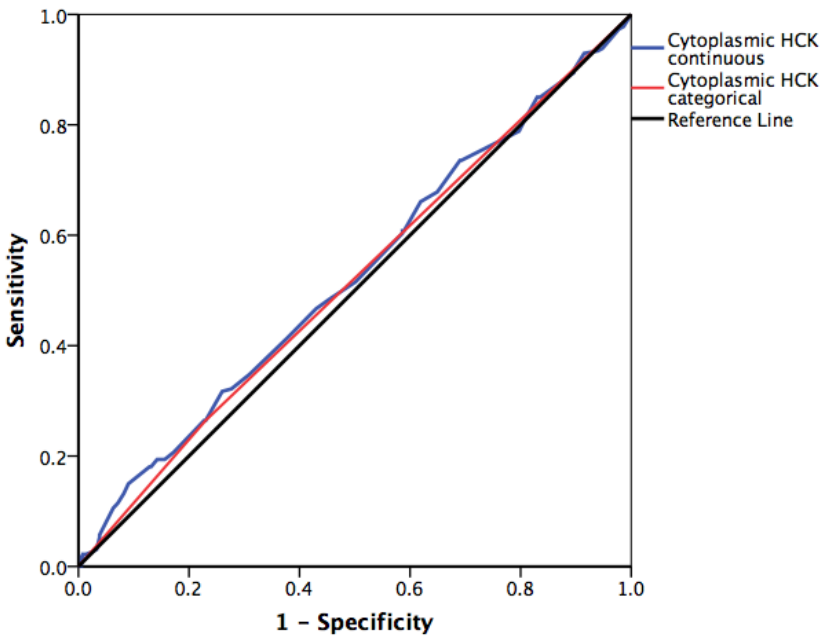


Supplementary figure 5.72: The distribution of cytoplasmic HCK measurements in patients stratified by survival status (p=0.295)



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	448	386	360	330	304	289	265
High	144	122	111	97	91	81	78

Supplementary figure 5.73: The relationship between cytoplasmic HCK expression and overall survival in patients with CI colorectal cancer



Supplementary figure 5.74: Predictive value of cytoplasmic HCK in identifying patients with CI colorectal cancer who will die during follow-up

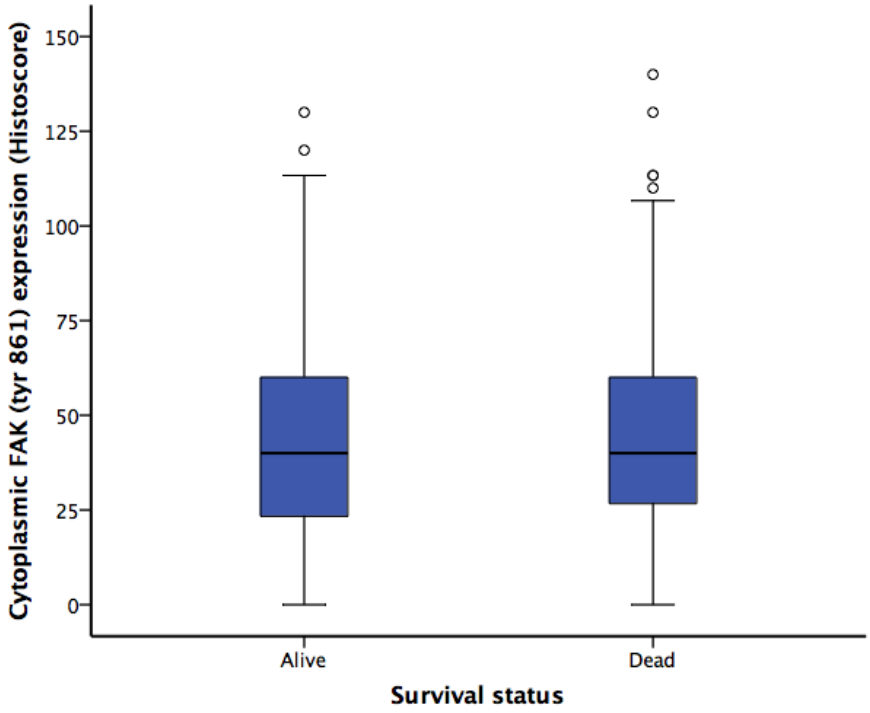
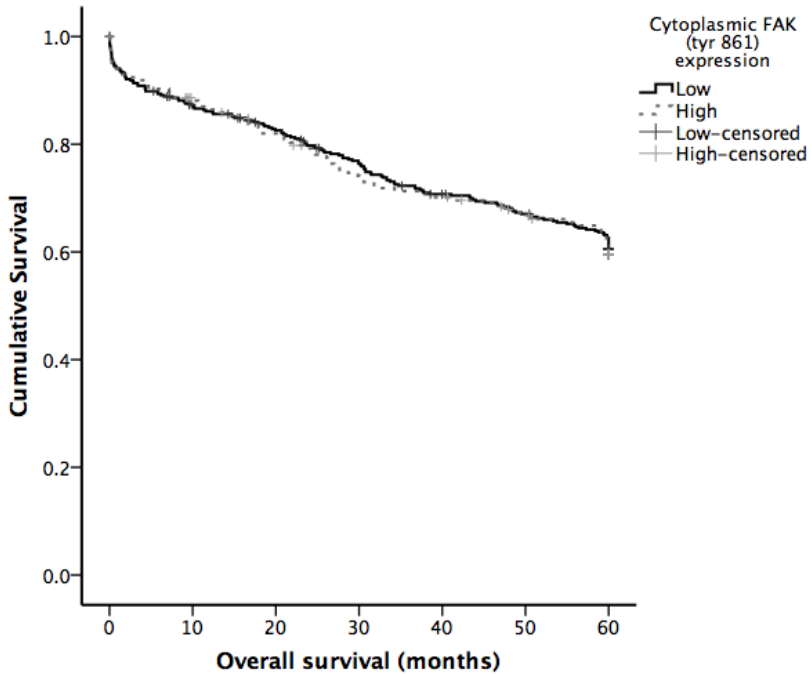
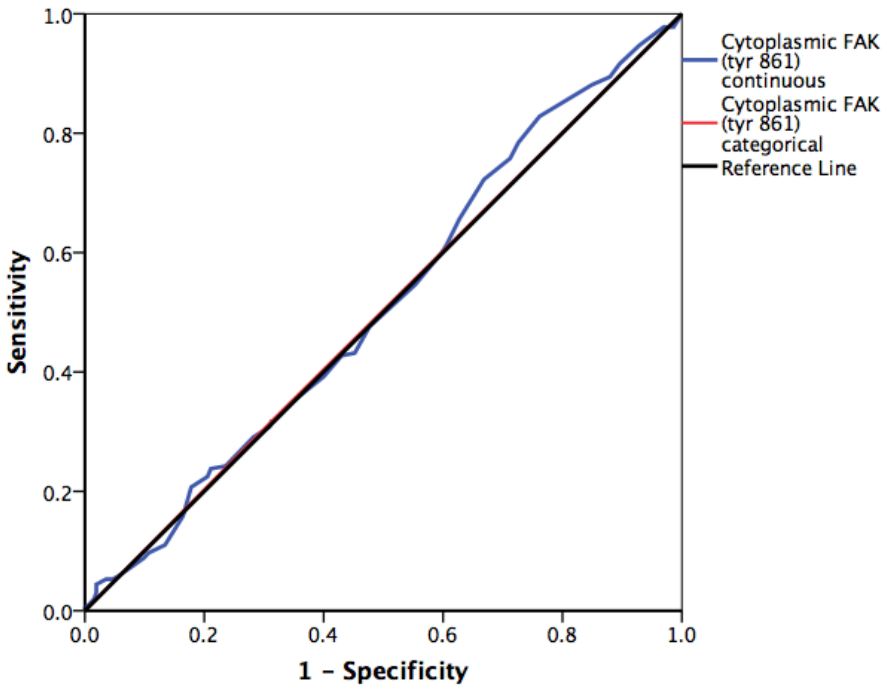


Figure 5.75: The distribution of cytoplasmic FAK (tyr 861) measurements in patients stratified by survival status (p=0.573)



No. at risk	0 months	10 months	20 months	30 months	40 months	50 months	60 months
Normal	406	348	324	296	271	256	238
Low	186	160	147	131	124	114	105

Supplementary figure 5.76: The relationship between cytoplasmic FAK (tyr 861) expression and overall survival in patients with CI colorectal cancer



Supplementary figure 5.77: Predictive value of cytoplasmic FAK (tyr 861) in identifying patients with CI colorectal cancer who will die during follow-up

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